Iron transport strategies of the genus *Burkholderia*

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Summary

The genus *Burkholderia* currently comprises more than 70 species that inhabit remarkably diverse niches ranging from soil and plant rhizosphere to the respiratory tract of cystic fibrosis patients. *Burkholderia* species, namely *B. cepacia* was first described as a plant pathogen causing sour skin in onions. Several *Burkholderia* species have exhibited beneficial interactions with their plant host and have been reported to have biocontrol and plant growth-promoting properties. However, some *Burkholderia* species are also human pathogens, including the primary human pathogens *B. pseudomallei* and *B. mallei* and opportunistic human pathogens such as 17 members of the *Burkholderia cepacia* complex (Bcc) group. Hence *Burkholderia* species in general is known to occupy several niches and may have pathogenic or beneficial interactions with plants and could be pathogenic to humans, making it a very versatile and adaptable group of organisms among the beta proteobacteria.

Iron is an important micronutrient for the growth of organisms, including *Burkholderia*, irrespective of their habitats. Since iron exists in the insoluble ferric form in oxic environments, bacteria have evolved various mechanisms to assimilate enough iron for all their vital processes. *B. cenocepacia*, a member of Bcc, is an important opportunistic pathogen in cystic fibrosis patients and its pathogenic traits have been extensively studied. Siderophores, which are iron-chelating molecules, have been shown to be important virulence factors in different infection models. However, our previous study on the importance of these siderophores in the growth of a clinical isolate, *B. cenocepacia* H111, revealed that siderophores are dispensable for the growth of this pathogen in iron limited conditions, even though the pathogenic potential of this strain was compromised. This finding suggested that *Burkholderia* could employ an alternative strategy to compensate for the defective siderophore systems. An investigation of the *Burkholderia* genome revealed a putative iron transport system and this study was initiated to identify and characterize this alternative iron transport strategy.

My work showed that *Burkholderia* possess a novel iron transport system, $Ftr_{Bcc}ABCD$, which is conserved in all sequenced *Burkholderia* species. The Ftr system is a high affinity iron transport system, which can transport iron in both ferric and

ferrous forms. Unlike siderophores, it is not critical for the virulence of the *Burkholderia* strain. A further screen of *Burkholderia* isolates for these different iron transport strategies revealed that some environmental *Burkholderia* isolates do not produce siderophores, while the Ftr system is a widely distributed trait among these strains. This finding prompted me to investigate the specific role of the Ftr system in other habitats and my results demonstrate that this novel system contributes more to the growth of *Burkholderia* strains in soil than siderophores. However, none of these systems were found to be essential for nodulation and the symbiotic association of *Burkholderia* strains with legumes.

In my investigations of the ecophysiology of siderophores in environmental *Burkholderia* strains, I introduced the ornibactin locus into 2 environmental *Burkholderia* isolates, which did not produce any siderophores. This locus encodes the biosynthesis and transport genes for the siderophore, ornibactin. Characterization of these engineered strains showed that introduction of this single virulence trait increases the pathogenic potential of *B. sacchari*, a soil *Burkholderia* isolate. Whether this is a strain-specific trait, or introduction of this virulence factor renders any harmless *Burkholderia* strain pathogenic, requires further elucidation.

In general, this study describes a novel iron transport system in *Burkholderia* species and demonstrates the specific roles of different iron transport systems in the diverse habitats occupied by this genus.

Zusammenfassung

Der Genus *Burkholderia* umfasst momentan mehr als 70 Spezies die in verschiedenen Nischen, wie etwa Erdböden, die Pflanzenrizosphere und die Lungen von Patienten mit zystischer Fibrose umfassen. Die Spezies *Burkholderia*, genauer gesagt, *B. Cenocepacia* wurde als Erstes als Pflanzenpathogen beschrieben, der die Krankheit „sour skin“ bei Zwiebeln verursacht. Verschiedenste *Burkholderien* haben aber auch vorteilhafte Interaktione wie zum Beispiel wachstumsfördernden Eigenschaften bei Pflanzen gezeigt. Neben diesen nützlichen *Burkholderien* gibt es jedoch auch humanpathogene Spezies. Diese umfassen primäre Humanpathogene wie *B. pseudomallei* und *B. mallei* und opportunistische Humanpathogene wie die 17 Mitglieder des *Burkholderia cepacia* complex (Bcc). Folglich sind *Burkholderien* bekannt dafür verschiedenste Umweltnischen zu besetzen und sowohl pathogene als auch nützliche Interaktionen mit Pflanzen zu haben. Dies macht sie zu äußerst vielseitigen und anpassungsfähigen Organismen aus der Gruppe der Beta-Proteobakterien.

Unabhängig von ihrem Lebensraum ist Eisen ein wichtiger Mikronährstoff für das Wachstum von Organismen wie *Burkholderien*. Eisen kommt in sauerstoffreichen Habitaten in seiner unlöslichen Form als Fe^{3+} vor. Daher haben Bakterien verschiedenste Mechanismen entwickelt um genügend Eisen für all ihre vitalen Prozesse aus diesen Habitaten zu assimilieren. *B. cenocepacia*, ein Miglied des Bcc, ist ein klinisch relevanter opportunistischer Pathogen in Patienten mit zystischer Fibrose dessen Pathogenitätsmechanismen eingehend studiert wurden. In verschiedenen Pathogenitätsmodellen wurde gezeigt, dass Siderophore, welche wichtige eisenkomplexierende Moleküle sind die von *B. cenocepacia* sekretiert werden, wichtige Virulenzfaktoren darstellen. Unsere vorangegangen Studie bezüglich der Bedeutung dieser Siderophore für das Wachstum des klinischen Isolates *B. cenocepacia* H111 hat jedoch gezeigt, dass Siderophore für das Wachstum in Eisen limitiertem Medium entbehrlich sind, wohingegen das Pathogenitätspotential verringert war. Dies lässt den Schluss zu, dass *Burkholderien*

möglicherweise eine alternative Strategie zur Eisenaufnahme besitzten, welche das Fehlen von Siderophoren kompensieren kann. Bei der Untersuchung mehrerer *Burkholderien* Genome konnten wir ein putatives Eisentransportsystem finden. Dies war der Startpunkt dieser Studie mit dem Ziel der Identifizierung und Charakterisierung dieses alternativen Eisentransportsystems.

Meine Arbeit zeigte, dass *Burkholderien* ein neuartiges Eisentransportsystem, Ftr_{Bcc}ABCD, besitzen, welches in allen sequenzierten *Burkholderia* Spezies vorhanden ist. Das Ftr System ist ein hoch affines Eisentransportsystem, welches Eisen in seiner Fe^{2+} und Fe^{3+} Form transportieren kann. Im Gegensatz zu den Siderophoren, ist das Ftr System nicht von entscheidender Bedeutung für die Virulenz der *Burkholderia* Stämme. Eine weitere Untersuchung unterschiedlicher *Burkholderien* Isolate zeigte, dass einige Umweltisolate zwar keine Siderophore produzieren, das Ftr System hingegen ein weit verbreitetes Merkmal in diesen Stämmen darstellt. Aufgrund dieses Ergebnisses untersuchte ich die spezifische Rolle des Ftr Systems in anderen Habitaten und meine Ergebnisse zeigen, dass dieses neuartige Eisentransportsystem für das Wachstum von *Burkholderia* Stämmen im Erdboden wichtiger ist als Siderophore. Es zeigte sich auch, dass weder das Ftr System noch Siderophore essentiell für die Nodulierung und die Symbiose von *Burkholderien* mit Leguminosen sind.Zur Erforschung der Ökophysiologie von Siderophoren in Umweltisolaten von *Burkholderia*, habe ich den genetischen Locus des Siderophores Ornibactin, welcher sowohl die Biosynthese- sowie die Transportergene für Ornibaction kodiert, in zwei *Burkholderia* Umweltisolate transferiert. Die Untersuchung dieser beiden genetisch veränderten Umweltisolate zeigte, dass das Pathogenitätspotential eines der beiden Stämme, *B. sacchari*, durch die Einführung dieses Virulenzfaktors erhöht wurde. Ob es sich hierbei um ein stammspezifisches Merkmal handelt, oder die Einführung von einzelnen Virulenzfaktoren in harmolse *Burkholderien* generell zu einer erhöhten Pathogenität führt, bleibt aber noch zu erörtern.

Diese Studie beschreibt ein neuartiges Eisentransportsystem in *Burkholderien* und demonstriert zusätzlich die spezifischen Rollen verschiedener Eisentransportsystem in diversen Habitaten die vom Genus *Burkholderia* besiedelt werden.

Abbreviations

°C	Degree Celsius
AHL	N-acyl-homoserine lactone
Amp	Ampicillin
Approx.	approximately
BDSF	Burkholderia diffusible signal factor, cis-2-dodecenoic acid
Bcc	Burkholderia cepacia complex
bp	Base pair
CAS	Chrome azurol S
cDNA	Complementary DNA
CF	Cystic fibrosis
CFU	Colony forming units
Cm	Chloramphenicol
ddH_2O	Double distilled water (Milli-Q water)
dH_2O	deionised water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
EPS	Exopolysaccharide
EtOH	Ethanol
g	Gram
Gm	Gentamicin
HDTMA	Hexadecyltrimethyl ammonium bromide

kb	kilobases
Kn	Kanamycin
kV	kilovolt
l	Liter
LB	Luria Bertani
M	Molar
mM	Millimolar
µM	micromolar
mg	milligram
µg	Microgram
ml	Milliliter
µl	Microliter
ng	Nanogram
NGM	nematode growth medium
nm	Nanometer
nM	Nanomolar
OD	optical density
ORF	Open reading frame
PBE	plant-beneficial-environmental
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
QS	quorum sensing
^{R}	Resistant
RNA	ribonucleic acid

rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Strep	Streptomycin
T1SS	type I secretion system
T2SS	type II secretion system
T3SS	type III secretion system
T4SS	type IV secretion system
T5SS	type V secretion system
T6SS	type VI secretion system
TLC	thin layer chromatography
Tp	Trimethoprim
w/v	weight per volume
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

Chapter 1: Introduction

1.1.Role and properties of iron in bacteria

Iron is an important transition metal and exists in two interconvertible ferric ($Fe3^{+}$) and ferrous ($Fe2^{+}$) redox states. This property makes iron a versatile catalyst in biological systems. The biologically relevant ferric and ferrous iron forms are incorporated into metalloproteins such as aconitase and cytochromes, which are necessary for oxidative metabolism in the tricarboxylic acid cycle. Iron is also found in many mono- and di-nuclear iron proteins like ferritin and ribonucleotide reductase. The redox potential of iron makes it especially useful for biological processes, in particular for oxidative phosphorylation where it facilitates electron transfer in the respiratory chain. Moreover, iron is present in different proteins with diverse functions that include DNA replication and repair, oxygen transport, carbon metabolism and regulation of gene expression (Messenger & Barclay, 1983).

Iron is very plentiful, being the fourth most abundant element on earth's crust. However, it exists in the insoluble ferric form in oxic environments at neutral pH and therefore its availability is growth limiting in various ecological niches (Andrews *et al*., 2003). In addition, excess iron is toxic due to the generation of reactive oxygen species in the presence of oxygen (Touati, 2000). Therefore all living cells including bacteria have evolved various iron transport strategies and homeostasis mechanisms to assimilate iron and regulate cellular iron concentrations.

1.2.Iron transport mechanisms in bacteria

Ferric iron has an extremely low solubility (10^{-18} M) at pH 7 and bacteria generally require around 10^{-7} to 10^{-5} M iron for optimal growth (Andrews *et al.*, 2003). Consequently, bacteria employ several mechanisms to solubilize and transport adequate iron. This assimilation is achieved mainly by three means; 1) production of chelators to scavenge ferric iron 2) reduction of insoluble ferric iron to soluble ferrous iron and 3) protonation, thus lowering the external pH to make ferric iron more soluble (Guerinot, 1994).

1.2.1 Iron chelation by siderophores

One of the most important mechanisms employed by organisms to solubilize iron is the production of chelators called siderophores. They are low moleculer weight, high affinity iron binding ligands that facilitate the solubilisation and transport of ferric iron into the cell (Crosa, 1989). The ferric siderophore complexes are recognized by specific receptors in the outer membrane and they are shuttled to the ABC transporters in the cytosolic membrane by periplasmic binding proteins and further channeled into the cytosol. This transport is mediated by the energy transducing TonB-ExbB-ExbD protein complex (Andrews *et al.*, 2003; Guerinot, 1994). Iron siderophore complexes are dissociated by reducation of ferric iron and siderophores are recycled by the cell (Figure 1).

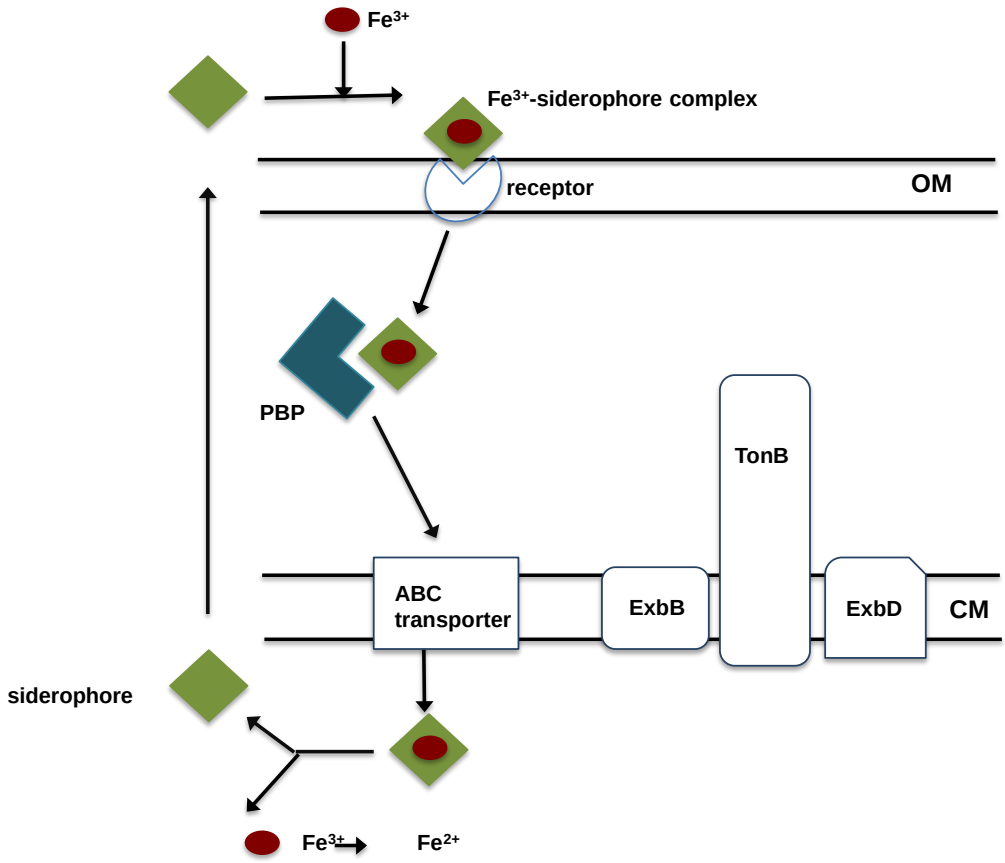

Figure 1: Siderophore mediated iron transport in gram negative bacteria (adapted from Andrews *et al.*, 2003 and Cornelis, 2010)

More than 500 siderophore types have been characterized which are classified according to the different functional groups of their iron ligands (Winkelmann, 1997). Although they show considerable structural variation, siderophores usually form hexadentate octahedral complexes with Fe^{3+} iron. The three major functional groups associated with these ligands are catechols, hydroxamates and α-hydoxycarboxylates, which all possess a high affinity for iron (Hider & Kong, 2010).

Siderophore type	**Siderophore**	**Producers**
Catecholate	Enterobactin	Enteric bacteria
	Vibriobactin	*Vibrio cholerae*
Phenolate	Yersiniabactin	*Yersinia* spp.
	Pyochelin	*Pseudomonas aeruginosa* *Burkholderia cepacia*
Hydroxamate	Alcaligin	*Alcaligenes dinitrificans* *Bordetella* spp.
	Ornibactin	*Burkholderia cepacia*
Carboxylate	Staphyloferrin A	*Staphylococcus* spp.
	Achromobactin	*Erwinia chrysantham*

Table 1: Major siderophore types, examples and their natural producers (adapted from Miethke & Marahiel, 2007)

1.2.2 Ferric reduction and transport of ferrous iron

Extracellular ferric reductase activity has been reported in some bacteria including *E. coli* and may be pivotal in reducing extracellular ferric iron to its ferrous form (Cowart, 2002). Many bacteria are also known to possess ferrous iron transport pathways, which can facilitate the transport of extracellular ferrous iron. The metal ABC transporter FutABCD, Zip transporter ZupT, *E. coli* EfeUOB system as well as the well-characterized FEO system are involved in ferrous iron transport (Katoh, 2001; Grass *et al*., 2005; Grosse *et al*., 2006; Kammler *et al*., 1993). Among these pathways, the Feo system is widely distributed across bacterial species and is particularly important under low oxygen conditions where ferrous iron remains stable (Hantke,

1987; Kammler *et al*., 1993). The recently characterized Ftr transport system in *Bordetella pertussis* and *Brucella abortus* are also proposed to transport ferrous iron (Brickman and Armstrong, 2012: Elhassanny *et al*., 2013).

1.2.3 ATP-binding Cassette (ABC) type metal transporters

ABC transporters for metals and low affinity iron transport systems have been reported in different bacteria. These ABC transport systems generally do not require outer membrane receptors or siderophores and instead depend on the periplasmic protein dependent ABC permeases to shuttle iron (Chakraborty *et al*., 2013). The Sfu system in *Serratia marcescens* transports ferric iron by employing an ABC permease independent of the TonB transport protein or membrane receptors (Zimmermann *et al*., 1989; Cornelis & Andrews, 2010). It has been suggested that iron bound to organic compounds are channeled through porins across the outer membrane (Angerer *et al*., 1992). Likewise, the VctPDGC transporter of *Vibrio cholerae* has been reported to transport siderophore free-iron ligands by means of PBP-dependent ABC transporters (Wyckoff & Payne, 2011). The SitABCD system of *Shigella flexneri* as well as *Salmonella enterica* and the homologous Yfe system in *Yersinia pestis* are similar systems, which have been shown to transport both forms of iron and are important for the growth of these bacteria in iron limited conditions (Runyen-Janecky *et al*., 2003; Zhou *et al*., 1999; Bearden & Perry, 1999).

1.3 Iron transport systems in different environments

A variety of iron transport systems have been characterized in bacteria and even closely related bacteria can employ different mechanisms to transport iron. This reflects the diverse niches occupied by such strains as well as the different sources of iron available in different environments.

1.3.1 Iron acquisition by pathogens

The process of withholding iron from infecting bacterial pathogens is one of the first lines of host defense (Kehl-Fie & Skaar, 2010). In mammals, the majority of iron is intracellular, and is sequestered by iron binding proteins such as lactoferrin, transferrin or ferritin to reduce its availability (Arber *et al*., 1978). Additionally, mammals produce proteins such as hemopexin and haptoglobin that bind heme and

hemoglobin thus limiting iron availability to pathogens (Andrews *et al*., 2003). Consequently, pathogens employ several mechanisms to evade this 'nutritional immunity'. With the exception of lactobacilli and *Borrelia burgdorferi*, that substitute iron with manganese in their metal requiring enzymes, other bacteria acquire iron through high affinity systems (Archibald, 1983; Posey, 2000). These transport systems include siderophore systems, heme uptake systems and lactoferrin/transferrin receptors.

1.3.1.1.Siderophore based transport systems

Pathogens are known to circumvent low iron availability by employing high affinity siderophore systems. Siderophores can bind iron with a high association constant which enables them to compete effectively with host iron binding proteins to sequester iron (Bullen & Griffiths, 1987). Siderophore systems have been shown to be essential for host colonization by several pathogenic strains and siderophore defective strains are reported to have attenuated virulence in many infection models (Ratledge & Dover, 2000; Bullen & Griffiths, 1987).

1.3.1.2.Heme acquisition systems

The majority of iron in the mammals is stored intracellularly in iron binding proteins such as ferritin and hemoglobin. Microorganisms generally employ two mechanisms to acquire iron from heme (Perkins-Balding *et al*., 2004; Wandersman & Delepelaire, 2004). The most common mechanism involves surface receptors that recognize and bind heme, or heme bound to proteins such as hemoglobin or hemopexin. The isolated heme is then transported across the outer membrane in a TonB-ExbB-ExbD dependent manner (Ratledge & Dover, 2000; Skaar, 2010). Some bacteria are known to secrete molecules called hemophores that can remove heme from host hemoproteins, which is then delivered to outer membrane heme receptors (Wandersman & Delepelaire, 2004). Heme is transported across the cytoplasmic membrane by means of ABC permeases and finally heme oxygenases degrade the heme, releasing complexed iron (Genco & Dixon, 2001).

1.3.1.3.Iron uptake from transferrin, lactoferrin and ferritin

The lactoferrins, serum transferrins and ovotransferrins together constitute a family of iron binding proteins termed lactoferrins. In addition to acquiring iron from these

compounds by means of siderophores, some bacteria such as *Neisseria* possess specific receptors that directly recognize these host proteins. Iron is stripped off from the lactoferrins and subsequently transported into the cell cytoplasm by means of a PBP ABC permease system (Andrews *et al*., 2003). The vast majority of iron in a vertebrate host is sequestered within the iron storage protein ferritin. It has been shown that some bacteria such as *B. cenocepacia* can acquire iron from this host iron storage protein. It is believed that proteases are involved in scavenging iron from this compound (Whitby *et al*., 2006).

1.3.2 Iron acquisition by plant-associated as well as soil microbes

Siderophores are proposed to be of high ecological significance in soils and on plant surfaces owing to their role as iron sources and biocontrol agents (Guerinot, 1994). These high affinity iron transport systems are assumed to be important for microbes in microenvironments where there is intense competition between bacteria, plants and fungi (Page, 2012). However, employing low affinity chelators such as organic acids or reducing agents to solubilize iron is a common strategy associated with soil microbes (Loper & Buyer, 1991).

Plant-associated *Rhizobia* have been shown to take up ferrous iron more efficiently than ferric iron. Siderophores and low affinity chelators such as citrate have also been shown to be important for nodulation and symbiosis between legumes and *Rhizobia* (Brear *et al*., 2013).

1.4 The genus *Burkholderia*

The genus *Burkholderia* comprises metabolically and ecologically diverse group of gram negative bacteria (Coenye & Vandamme, 2003; Mahenthiralingam *et al*., 2005; Sawana *et al*., 2014). They inhabit a diverse range of ecological niches ranging from soil and the plant rhizosphere to the respiratory tract of CF patients (Coenye & Vandamme, 2003; Janssen, 2006). A group of 17 phylogenetically similar, but genetically distinct *Burkholderia* species, the *Burkholderia cepacia* complex (BCC), is mainly associated with respiratory infections in cystic fibrosis patients (Mahenthiralingam *et al*., 2005; Hauser *et al*., 2011). These strains also exhibit an

extraordinary metabolic versatility allowing them to adapt to a wide range of environments. *B. pseudomallei* and *B. mallei* are related to the Bcc, and are responsible for potentially lethal infections in humans and animals respectively (Limmathurotsakul & Peacock, 2011; Whitlock *et al*., 2007). These pathogens, along with the plant pathogens *B. glumae* and *B. gladioli*, constitute the pathogenic clade of *Burkholderia* (Suárez-Moreno *et al*., 2012). The other cluster contains non-pathogenic *Burkholderia* species, many of which are beneficial to plants. This cluster includes legume symbionts that can fix atmospheric nitrogen, plant growth promoters and phytoremediation as well as biocontrol agents. They are known as the plant-beneficial-environmental (PBE) cluster (Figure 2) (Suárez-Moreno *et al*., 2012).

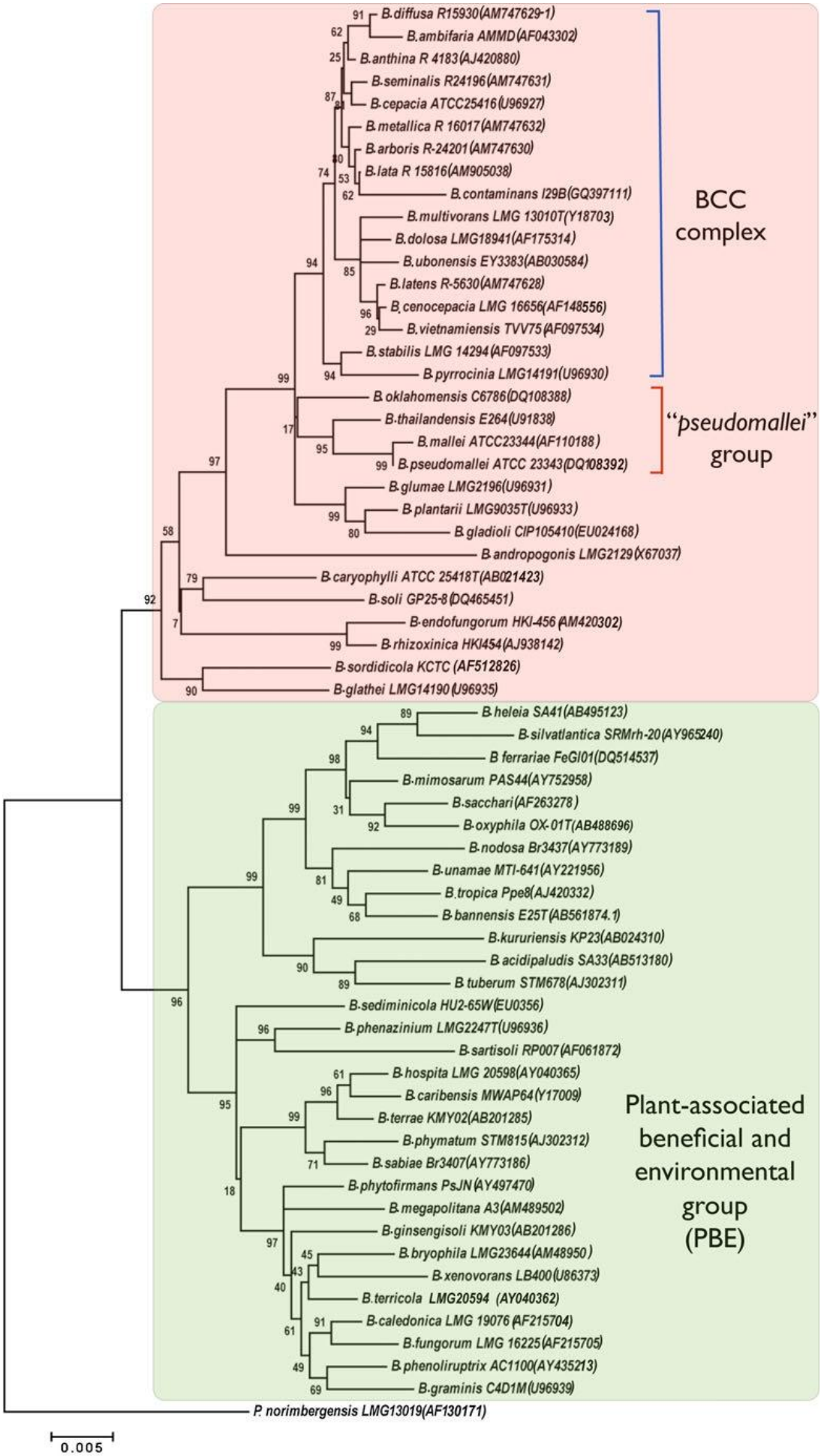
B.diffusa R15930(AM747629-1)
B.ambifaria AMMD(AF043302)
B.anthina R 4183(AJ420880)
B.seminalis R24196(AM747631)
B.cepacia ATCC25416(U96927)
B.metallica R 16017(AM747632)
B.arboris R-24201(AM747630)
B.lata R 15816(AM905038)
B.contaminans I29B(GQ397111)
B.multivorans LMG 13010T(Y18703)
B.dolosa LMG18941(AF175314)
B.ubonensis EY3383(AB030584)
B.latens R-5630(AM747628)
B.cenocepacia LMG 16656(AF148556)
B.vietnamiensis TVV75(AF097534)
B.stabilis LMG 14294(AF097533)
B.pyrrocinia LMG14191(U96930)
BCC complex
B.oklahomensis C6786(DQ108388)
B.thailandensis E264(U91838)
B.mallei ATCC23344(AF110188)
B.pseudomallei ATCC 23343(DQ108392)
"pseudomallei" group
B.glumae LMG2196(U96931)
B.plantarii LMG9035T(U96933)
B.gladioli CIP105410(EU024168)
B.andropogonis LMG2129(X67037)
B.caryophylli ATCC 25418T(AB021423)
B.soli GP25-8(DQ465451)
B.endofungorum HKI-456(AM420302)
B.rhizoxinica HKI454(AJ938142)
B.sordidicola KCTC (AF512826)
B.glathei LMG14190(U96935)
B.heleia SA41(AB495123)
B.silvatlantica SRMrh-20(AY965240)
B ferrariae FeGl01(DQ514537)
B.mimosarum PAS44(AY752958)
B.sacchari(AF263278)
B.oxyphila OX-01T(AB488696)
B.nodosa Br3437(AY773189)
B.unamae MTI-641(AY221956)
B.tropica Ppe8(AJ420332)
B.bannensis E25T(AB561874.1)
B.kururiensis KP23(AB024310)
B.acidipaludis SA33(AB513180)
B.tuberum STM678(AJ302311)
B.sediminicola HU2-65W(EU0356)
B.phenazinium LMG2247T(U96936)
B.sartisoli RP007(AF061872)
B.hospita LMG 20598(AY040365)
B.caribensis MWAP64(Y17009)
B.terrae KMY02(AB201285)
B.phymatum STM815(AJ302312)
B.sabiae Br3407(AY773186)
B.phytofirmans PsJN(AY497470)
B.megapolitana A3(AM489502)
B.ginsengisoli KMY03(AB201286)
B.bryophila LMG23644(AM48950)
B.xenovorans LB400(U86373)
B.terricola LMG20594 (AY040362)
B.caledonica LMG 19076(AF215704)
B.fungorum LMG 16225(AF215705)
B.phenoliruptrix AC1100(AY435213)
B.graminis C4D1M(U96939)
Plant-associated beneficial and environmental group (PBE)
P. norimbergensis LMG13019(AF130171)
0.005

Figure 2: Phylogenetic tree based on 16S rRNA gene sequences of the recognised species of the *Burkholderia* genus: from Suárez-Moreno *et al.*, 2012. . Red—the pathogenic *Burkholderia* clade; green—plant-associated beneficial and environmental (PBE) group

1.4.1 The Bcc as human opportunistic pathogens

Members of the Bcc are ubiquitously distributed in nature and have been isolated from a wide range of niches such as soil, the plant rhizosphere, industrial settings, hospital environments and the lungs of immunocompromised patients (Coenye & Vandamme, 2003). While some Bcc strains have exhibited potential biotechnological applications such as plant growth promotion and biocontrol properties, they are also opportunistic pathogens causing chronic lung infections in cystic fibrosis patients (Coenye and Vandamme, 2003; Eberl & Tümmler, 2004; Mahenthiralingam *et al*., 2008). Among the Bcc species, *B. multivorans* and *B. cenocepacia* are most commonly isolated from CF patients (Coenye & Vandamme, 2003; Mahenthiralingam *et al*., 2008). *B. cenocepacia* is also associated with epidemic spread between patients, intrinsic resistance to multiple antibiotics and cause fatal fulminant pneumonia in CF patients. For these reasons, *B. cenocepacia* has been the focus of research among *Burkholderia* species, which has resulted in the identification of several virulence determinants of this pathogen, which are described below.

1.4.1.1. Quorum sensing systems

Quorum sensing (QS) is the cell density dependent regulatory mechanism employed by bacteria to regulate gene expression by means of diffusible signal molecules. All Bcc species possess at least one classical LuxIR quorum sensing system termed the CepIR system (Venturi *et al*., 2004). The CepIR system, which mediates the production of N-acyl homoserine lactone, regulates the expression of various virulence factors such as extracellular proteases, lipases and siderophores (Lewenza *et al*., 1999). In addition, this system is also required for swarming motility (Lewenza *et al*., 2002), biofilm formation (Tomlin *et al*., 2005), virulence in mammalian infection models (Sokol *et al*., 2003) and killing of *C. elegans* (Köthe *et al*., 2003).

Besides the CepIR system, *Burkholderia* species are also known to possess a QS system that utilizes a chemical signal termed cis-2-dodecenoic acid, referred to as *Burkholderia* diffusible signal factor (BDSF) (Deng *et al*., 2009). BDSF has also been shown to regulate functions such as motility, EPS production, protease activity, biofilm formation and virulence in invertebrate models (Deng *et al*., 2012; Udine *et al*., 2013; Ryan *et al*., 2009).

1.4.1.2. Biofilms

Another important feature of the Bcc is the ability to form biofilms, which are complex multicellular bacterial communities that protect resident bacteria from antibiotics and host immune system defense mechanisms. Members of Bcc have been shown to form biofilms in lungs of CF patients as well as *in vitro* (Riedel *et al*., 2001; Conway *et al*., 2002). Several factors such as quorum sensing, EPS production, motility and iron availability have been shown to affect biofilm formation in the Bcc (Tomlin *et al*., 2005; Cunha *et al*., 2004; Huber *et al*., 2002; Berlutti *et al*., 2005). These findings indicate that biofilm formation is a complex process in the Bcc involving many of its virulence determinants. Moreover, Bcc bacteria growing in biofilms are reported to be more resistant to antibiotics than planktonic cells, resulting in better persistence of these strains in CF lungs (Caraher *et al*., 2007).

1.4.1.3. Protein secretion systems

Bacteria employ several secretion systems to deliver proteins or effector molecules important for virulence, which influence host cellular responses. *B. cenocepacia* and *B. vietnamiensis* have been found to have type I and II secretion systems which secrete proteins with hemolytic activity (Fehlner-Gardiner *et al*., 2002; Whitby *et al*., 2006). *B. cenocepacia* also has a type III secretion system which was reported to be important for virulence in a murine model of infection (Tomich *et al*., 2003). Two type IV secretion systems have been identified in *Burkholderia* species. One is responsible for secreting cytotoxic proteins, causing disease in onions and important for intracellular survival in phagocytes (Engledow *et al*., 2004; Sajjan *et al*., 2008), while the other plays a role in DNA mobilization (Zhang *et al*., 2009). A type VI secretion system has also been identified in *B. cenocepacia* and was shown to play a role in virulence in a murine infection model (Hunt *et al*., 2004).

1.4.1.4. Extracellular proteins such as proteases and lipases

Extracellular lipases and proteases are proposed to be involved in interaction of Bcc species with host epithelial cells (McClean & Callaghan, 2009). Metalloproteases and serine proteases are produced by many Bcc species and are thought to be involved in proteolysis of the extracellular matrix (Kooi *et al*., 2006). Two extracellular zinc metalloproteases, ZmpA and ZmpB were shown to be important for virulence of some *Burkholderia* strains in a rat agar bead model (Corbett *et al*., 2003; Kooi *et al*., 2006). Lipase production is also a trait widely distributed among *Burkholderia* species and was shown to be important for invasion of epithelial cells (Mullen *et al*., 2007).

1.4.1.5. Lipopolysaccharide, flagella and pili

Lipopolysaccharide (LPS) is a complex glycolipid present in the outer membrane of gram negative bacteria and the LPS of the Bcc has been shown to induce a strong immune response resulting in host cell damage (Hutchison *et al*., 2000). LPS mutants of Bcc species were also reported to be attenuated in virulence in infection models such as rat agar bead, *C. elegans* and *G. mellonella* (Loutet *et al*., 2006; Ortega *et al.*, 2009; Uehlinger *et al*., 2009). Disruption of another surface structure, the flagellum, resulted in an avirulent strain of *B. cenocepacia* unable to infect the rat agar bead model (Urban *et al*., 2004). Moreover, cable pili have also been shown to initiate cytoxicity in host epithelial cells (Cheung *et al*., 2007).

1.4.1.6. Exopolysaccharide (EPSs)

Exopolysaccharides are branched polysaccharide subunits secreted by bacteria. Many Bcc clinical isolates are reported to produce an EPS named cepacian (Cunha *et al*., 2004). Bcc mutants defective in cepacian formation have been shown to be avirulent in a mouse lung infection model, demonstrating the importance of EPS as a virulence factor (Sousa *et al*., 2007). However, some virulent members of the species, such as *B. cenocepacia* J2315 and K56-2, are nonmucoid denoting little or no production of EPS (Zlosnik *et al*., 2008).

1.4.1.7. Iron transport systems

Bacteria employ different mechanisms to overcome iron limitation in the host environment and these systems are extremely important for pathogens to thrive in the host. Members of the Bcc are known to utilize four siderophores, namely

pyochelin, ornibactin, cepabactin and cepaciachelin to solubilize and transport iron (Thomas, 2007). Ornibactin is the predominant siderophore produced by Bcc species and this siderophore has been shown to be critical for virulence of *B. cenocepacia* (Sokol *et al*., 1999; Visser *et al*., 2004; Uehlinger *et al*., 2009). However, a wide range of iron transport mechanisms have been described in *Burkholderia* species and a short description of these systems are given below.

1.4.2 Iron transport systems in *Burkholderia*

1.4.2.1. Siderophore systems

1.4.2.1.1. Pyochelin

Pyochelin is a structurally unique siderophore of the phenolate class, which is a condensation product of salicylic acid and two molecules of cysteine (Cox *et al*., 1981; Thomas, 2007). They exist as two easily interconvertible stereoisomers, pyochelin I and II (Schlegel *et al*., 2004) and bind iron with a stoichiometry of two pyochelin molecules per ferric iron (Tseng *et al*., 2006). In previous studies involving *B. cepecia* isolates from CF patients, approximately 50 % of strains did not produce any pyochelin, including the pathogenic *B. cenocepacia* K56-2 (Sokol, 1986). It has also been shown that pyochelin is less important for virulence of *B. cenocepacia in vivo* in different infection models (Visser *et al*., 2004; Mathew *et al*., 2014).

1.4.2.1.2. Ornibactin

Ornibactins are linear hydroxamate/hydroxycarboxylate siderophores similar to pyoverdin of *P. aeruginosa*, but lacking a chromophore (Stephan *et al*., 1993). In a previous screen of clinical Bcc isolates for production of siderophores, the majority of isolates produced ornibactin, illustrating the importance of this siderophore in iron transport and growth of Bcc strains (Darling *et al*., 1998). In fact malleobactin, an important siderophore produced by *B. pseudomallei*, is similar to ornibactin in structure and biosynthesis mechanism (Thomas, 2007; Franke *et al*., 2013). Previous studies have also shown that the ferric-ornibactin uptake system is important for iron uptake in chronic lung infections in model organisms and thus contributes significantly to the virulence of this pathogen (Visser *et al*., 2004) .

1.4.2.1.3. Cepabactin and cepaciachelin

Cepabactin is a cyclic hydroxamate, which has been shown to facilitate iron transport and stimulate growth of *B. cepacia* under iron-limited conditions (Meyer *et al.*, 1989). However in a previous screen of Bcc clinical isolates for siderophore production, only 10 % of the strains produced cepabactin (Darling *et al*., 1998).

Cepaciachelin is a catecholate siderophore identified in a rhizosphere isolate, *B. ambifaria* (Barelmann *et al*., 1996). It has not been reported in other *Burkholderia* strains and its role in iron transport or growth of *Burkholderia* strains has not been demonstrated.

1.4.2.2. Heme transport system

Burkholderia species are known to utilize heme as an iron source (Whitby *et al.*, 2006; Thomas, 2007). An ortholog of the heme transport, the Bhu system, has been identified in *B. cenocepacia* (Thomas, 2007). However, the role of this *bhu* operon in heme transport has not been experimentally demonstrated.

1.4.2.3. Ferritin transport

It has been reported that *B. cenocepacia* can utilize ferritin as an iron source (Whitby *et al*., 2006). Ferritin concentrations were shown to be higher in sputum and lungs of CF patients (Stites *et al.*, 1998) and hence ferritin is considered as a potential source of iron in this condition. Whitby *et al* proposed that serine proteases are involved in degrading ferritin making it available for uptake by *B. cenocepacia* (Whitby *et al*., 2006)

1.5 Preliminary results leading to this study

In my previous work investigating the role of siderophores in growth and virulence of *B. cenocepacia*, I observed that a *B. cenocepacia* mutant defective in pyochelin and ornibactin production did not exhibit any significant growth defect in iron limited conditions. This finding clearly suggested that this strain possesses an alternative high-affinity iron transport system that can compensate for defective siderophore synthesis. The presence of any other siderophores was ruled out using CAS assay. Based on these findings, two major questions arose:

1. What is the alternative, high affinity, iron transport system in *Burkholderia* species?

2. Are the different iron transport systems present in Burkholderia species redundant or essential under different conditions?

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Chapter 2. Objective of the study

Previous findings on the dispensability of siderophore systems in *Burkholderia* prompted us to investigate the *B. cenocepacia* genome in detail to look for potential iron transport systems. We identified a putative iron transport locus BCAE1568-1571 (subsequently named *ftrABCD*) that was conserved in all *Burkholderia* species. BLAST analysis revealed that the *ftr* locus was homologous to the main iron transport complex Fet3p/Ftr1 in *Saccharomyces cerevisiae* (Severance *et al*., 2004). This study was initiated to investigate and characterize this putative iron transport system in the model strain *B. cenocepacia* H111. In the first part of my work, I verified and studied the importance of the Ftr system in iron transport in *B. cenocepacia* H111. In the course of this work, the Ftr system was shown to be an alternative high affinity system important for the growth of this bacterium under iron limiting conditions. The second part of my work deals with elucidating the specific roles of siderophores and the Ftr system in different niches inhabited by *Burkholderia* species. In addition to looking at the importance of these systems in iron transport, alternative roles of *Burkholderia* siderophores in metal homeostasis were also investigated.

3. Results I

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Chapter 3: Results I. A novel siderophore-independent strategy of iron uptake in the genus *Burkholderia*

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3.1 Abstract

Like many other bacteria, *Burkholderia* sp. takes up iron in its ferric form *via* siderophore-dependent transporters. We observed that mutant strains of *B. cenocepacia* H111 unable to synthesize siderophores did not exhibit any growth defect under iron-limited conditions. This finding suggested that this opportunistic pathogen can adopt an alternative iron uptake strategy to compensate for the loss of siderophores. We identified a putative iron uptake locus, $ftr_{Bcc}ABCD$, in the genome of *B. cenocepacia* H111, which is also conserved in other members of the genus *Burkholderia*. Mutants deficient in both siderophore-dependent and $Ftr_{Bcc}ABCD$ systems failed to grow under iron-limited conditions and radiolabeled iron transport assays showed that these mutants were impaired in iron uptake. In addition, expression of $ftr_{Bcc}ABCD$ can restore growth of an *E. coli* strain lacking all known high-affinity iron transport systems under iron-limited conditions. We show that all four proteins encoded by the $ftr_{Bcc}ABCD$ are essential for iron uptake. Furthermore, our results indicate that the expression of $ftr_{Bcc}ABCD$ is regulated at the transcriptional level by iron concentration. This study provides evidence of an alternative, siderophore-independent, iron uptake system in *Burkholderia* species.

3.2 Introduction

The genus *Burkholderia* comprises a diverse group of β-proteobacteria with more than 70 described species that thrive in various ecological niches (Coenye and Vandamme, 2003; Mahenthiralingam *et al*., 2005; Suárez-Moreno *et al*., 2012). While *B. mallei* and *B. pseudomallei* are primary animal and human pathogens, some members of the genus have unique metabolic features which make them useful as biofertilizers and bioremediation agents (Coenye and Vandamme, 2003). Recently, several *Burkholderia* species came to prominence because of their ability to form nitrogen-fixing symbiotic associations with legumes (Suárez-Moreno *et al*., 2012). One phylogenetic clade, the *Burkholderia cepacia* complex (Bcc), is a heterogenous group consisting of 17 formally named species that are phenotypically similar but genetically distinct (Mahenthiralingam *et al*., 2000; Vermis *et al*., 2002; Vanlaere *et*

al., 2008; Vanlaere *et al.*, 2009). BCC strains have been isolated from soil, fresh water and sediments, the plant rhizosphere, industrial settings, and hospital environments (Mahenthiralingam *et al*., 2006). Bcc members have emerged as important respiratory pathogens for patients with cystic fibrosis or chronic granulomatous disease (Govan and Deretic, 1996). Among the 17 species of the complex, *Burkholderia multivorans* and *Burkholderia cenocepacia* are most frequently isolated from CF patients (LiPuma *et al*., 2001; Reik *et al*., 2005). Nevertheless, all members of the Bcc have been isolated both from the environment and from CF patient sputum, reflecting their ubiquitous distribution (Baldwin *et al*., 2007).

Since iron is a limiting nutrient in many habitats, *Burkholderia* species have evolved a wide range of strategies to overcome iron shortage and to ensure sufficient uptake. The most common systems rely on the synthesis and secretion of low molecular weight, high affinity iron binding siderophores (Guerinot, 1994; Neilands, 1995). Some *Burkholderia* strains are also capable of acquiring iron from heme and ferritin (Whitby *et al*., 2006).

Siderophore-mediated iron acquisition has been identified as an important virulence factor in members of the genus *Burkholderia* and was extensively studied in clinical isolates of *B. cenocepacia* (Thomas, 2007). They often produce two siderophores, pyochelin and ornibactin (Darling *et al*., 1998; Sokol *et al*., 1999) and these have been correlated with the ability of these strains to establish and maintain an infection (Sokol *et al*., 2000; Visser *et al*., 2004; Uehlinger *et al*., 2009). Previous studies have also shown that a single functional siderophore system is sufficient for growth of *B. cenocepacia* Pc715j in iron restricted conditions (Visser *et al*., 2004). However, little is known about the essentiality of the siderophore systems or alternative iron transport pathways in members of the genus *Burkholderia*. We have previously noted that genes encoding siderophores were absent in the plant symbiont *Candidatus* Burkholderia kirkii (Carlier and Eberl, 2012) and Agnoli and co-workers have previously reported that a spontaneous Bcc mutant strain lacking siderophores grew in medium supplemented with iron chelators, albeit with slightly slower growth rate than the parental strain (Agnoli *et al.*, 2006). Together, these

observations suggest the presence of an unidentified, alternative iron acquisition system in the genus *Burkholderia*.

Here we demonstrate that a novel ferric iron uptake pathway is encoded by *ftr$_{Bcc}$ABCD* of *B. cenocepacia* H111. Ftr$_{Bcc}$ABCD is homologous to members of a novel family of siderophore-independent iron uptake machineries that exhibit similarities to the well characterized fungal Fet3p-Ftr1p system (Stearman *et al*., 1996; Severance *et al*., 2004). The EfeUOB ferrous iron transporter in *Escherichia coli* (Grosse *et al*., 2006; Cao *et al*.,2007), the EfeUOB ferric and ferrous iron transporter in *Bacillus subtilis* (Miethke *et al*., 2013), the FetMP system in *E. coli* (Koch *et al*., 2011) and the FtrABCD ferrous iron transporter in *Bordetalla pertussis* (Brickman and Armstrong, 2012) and *Brucella abortus* (Elhassanny *et al*., 2013) belong to this transporter family and share structural as well as functional domains. The *ftr$_{Bcc}$ABCD* gene cluster is conserved across the genus *Burkholderia* and we show that it can compensate for the absence of siderophore systems. The components of the Ftr$_{Bcc}$ABCD system share approximately 50% identity to the recently described Ftr systems in *B. pertussis* (Brickman and Armstrong, 2012) and *B. abortus* (Elhassanny *et al*., 2013), which were shown to transport ferrous iron and are important for growth under iron-restricted conditions at acidic pH (Brickman and Armstrong, 2012; Elhassanny *et al*., 2013). In addition, iron transport by FtrABCD was shown to be important for virulence of *B. abortus* (Elhassanny *et al*., 2013). Although the structural components of the Ftr$_{Bcc}$ABCD system are homologous to those of the FtrABCD system, we present evidence that it functions as a ferric iron transporter and is not critical for pathogenicity.

3.3 Results

3.3.1 Both pyochelin and ornibactin are dispensible for the growth of *B. cenocepacia* H111

To investigate the importance of siderophore-based iron uptake systems in *Burkholderia* species, we constructed null mutants of our model strain *B. cenocepacia* H111 that are defective in the biosynthesis of either pyochelin or ornibactin or both siderophores. The production of extracellular iron chelators in

these mutants was assessed using the Chrome Azurol S (CAS) assay. H111 and the single siderophore mutants, BccAM01 (ornibactin-negative) and BccAM02 (pyochelin-negative), displayed well defined, pale halos around the colonies, characteristic of either pyochelin or ornibactin production. The absence of a halo around colonies of the double mutant BccAM03 indicated a complete lack of siderophore activity even after 72 hours of incubation (Figure 3A).

To determine whether a functional siderophore system is required for optimal growth in iron-limited conditions, we measured the growth rates of our set of siderophore mutants and the wild-type strain in iron-limited succinate (IFS) medium. As shown in figure 3B, inactivation of pyochelin or ornibactin biosynthesis genes did not affect growth in iron-limited conditions. Even the double mutant, BccAM03, which is unable to acquire iron through uptake of siderophores, grew in the iron-restricted medium with a growth rate similar to that of the wild type, although the strain exhibited a slightly longer lag phase (Figure 3B). Growth of the double mutant strain BccAM03 was slightly inhibited only when a strong iron chelator such as 2-2' dipyridyl was added to the growth medium (Figure S1). This demonstrates that the production of pyochelin and ornibactin is not required for optimal growth of H111.

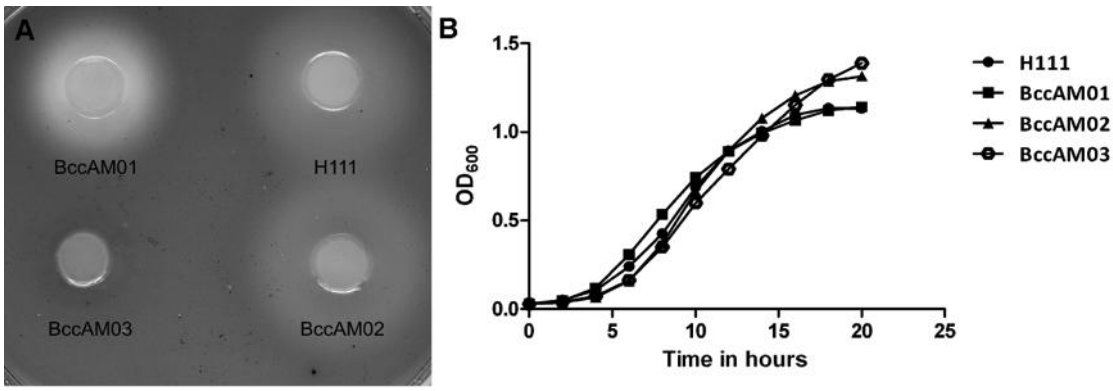


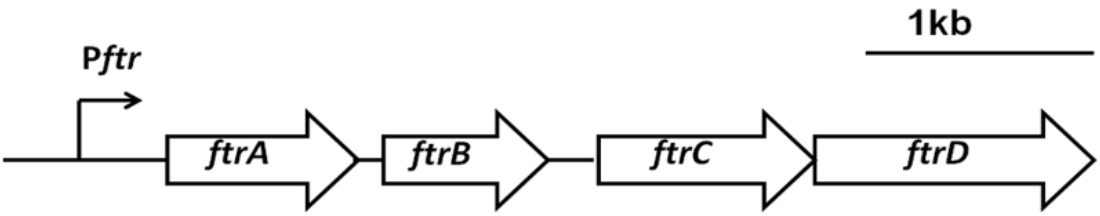
Figure 3: Role of siderophores in the growth of *B. cenocepacia* H111 (A) Siderophore production on CAS agar by H111, BccAM01, BccAM02 and BccAM03 after 48 hours of incubation. A discolored halo surrounding the bacterial colonies indicates siderophore production. Note the absence of a halo around the BccAM03 colony, suggesting that this strain does not produce any siderophores. (B) Growth curves of *B. cenocepacia* H111 (black circles), BccAM01 (black squares), BccAM02 (black triangles) and BccAM03 (white circles) in IFS medium. Error bars represent the standard error of three independent experiments.

3.3.2 A novel iron uptake system in *Burkholderia*

The ability of the siderophore mutants of H111 to grow in iron-limited conditions suggested the presence of an alternative iron uptake system. This prompted us to search the H111 genome annotation for putative iron transport systems. We identified a locus comprised of 4 open reading frames, which we named *ftr$_{Bcc}$ABCD* (Locus tag: I35_4016 – I35_4019; Figure 4A). Ftr$_{Bcc}$ABCD shares more than 50% sequence identity with the recently described FtrABCD system of *B. pertussis* (Brickman and Armstrong, 2012) and *B. abortus* (Elhassanny *et al*., 2013). The putative functions of the four components of the locus, as well as homologs of the predicted products are shown in Figure 4B. The predicted Ftr$_{Bcc}$C protein is a putative iron permease belonging to the FTR1 family of integral membrane iron transporters (Kosman, 2003). The best characterised member of this protein family is FTR1 of *Saccharomyces cerevisiae*, which interacts with a multicopper oxidase and mediates high-affinity iron uptake (Stearman *et al*., 1996). Sequence alignment shows that Ftr$_{Bcc}$C shares 64% and 67% identity to the FtrC protein of *B. abortus* and *B. pertussis,* respectively. Two REXXE motifs, shown to be essential for ferric iron trafficking in the yeast FTR1 transporter (Stearman *et al*., 1996; Severance *et al*., 2004), are located on the first and fourth transmembrane domains of Ftr$_{Bcc}$C as in the yeast and bacterial homologs.

The *ftr$_{Bcc}$B* gene, located immediately upstream of *ftr$_{Bcc}$C*, encodes a putative periplasmic cupredoxin which is homologous to FtrB proteins and EfeO, the cupredoxin component of the EfeUOB system of *E. Coli* (Rajasekaran *et al.*, 2010). Adjacent to *ftr$_{Bcc}$B* in H111 is *ftr$_{Bcc}$A*, which encodes a putative 19kDa periplasmic protein similar to FtrA (Brickman and Armstrong, 2012; Elhassanny *et al*., 2013), the P19 protein of *Campylobacter jejuni* (Chan *et al*., 2010) and FetP protein of the FetMP system in *E. coli* (Koch *et al*., 2011). FetP of *E. coli* enhanced iron uptake at low ferrous iron concentrations and at higher ferric iron concentrations. This has been attributed to its dual role as a periplasmic ferrous iron binding protein and a ferric reductase (Koch *et al*., 2011). The fourth component of *ftr$_{Bcc}$ABCD* operon is the Ftr$_{Bcc}$D protein, which is a putative ferredoxin sharing approximately 50% sequence identity with the homologous FtrD proteins.

A



B

ORFs in the locus	Putative function	Homologs
$ftr_{Bcc}A$	Periplasmic protein involved in high affinity ferrous iron transport	P19 in *C. jejuni* *fetP* in *E. coli* *ftrA* in *B. pertussis* and *B. abortus*
$ftr_{Bcc}B$	Cupredoxin	*efeO* in *E. coli* *ftrB* in *B. pertussis* and *B. abortus*
$ftr_{Bcc}C$	High affinity iron permease	*ftr1* in *S. cerevisiae* *efeU* in *E. coli* *ftrC* in *B. pertussis* and *B. abortus*
$ftr_{Bcc}D$	Ferredoxin	*ftrD* in *B. pertussis* and *B. abortus*

Figure 4: The f*trABCD* locus of *B. cenocepacia* H111. (A) Genetic organization of the $ftr_{Bcc}ABCD$ locus in *Burkholderia cenocepacia* H111. (B) Putative functions of the predicted products and their homologs.

3.3.3 A siderophore null mutant of H111 depends on the Ftr_{Bcc}ABCD system to grow in iron limited conditions

To test whether $ftr_{Bcc}ABCD$ encodes an iron uptake system, we generated an $ftr_{Bcc}C$ conditional mutant in the H111 wild-type strain and the siderophore double mutant BccAM03. In the resulting mutant strains, named BccAM04 and BccAM05, respectively, expression of $ftr_{Bcc}CD$ is dependent upon supplementation of the growth medium with rhamnose. While BccAM04 did not exhibit any growth defect in iron-limited conditions, growth of BccAM05 was severely impaired in IFS medium at pH 7 (Figure 5A). Growth of BccAM03 was reduced compared to BccAM04 when 60 µM of 2-2' dipyridyl was added to the growth medium, indicating an advantage of the siderophores uptake system over Ftr_{Bcc} at high concentrations of iron chelating agents (Figure S1). Supplementing the medium with rhamnose restored growth of strain BccAM05, showing that the inability of the mutant to grow under low iron

availability can be attributed to a defect in the expression of *ftr*$_{Bcc}$*CD* (Figure 3.3B). Introduction of hemin also rescued the growth of BccAM05, demonstrating that the growth defect of the strain was indeed due to iron limitation (Figure 7).

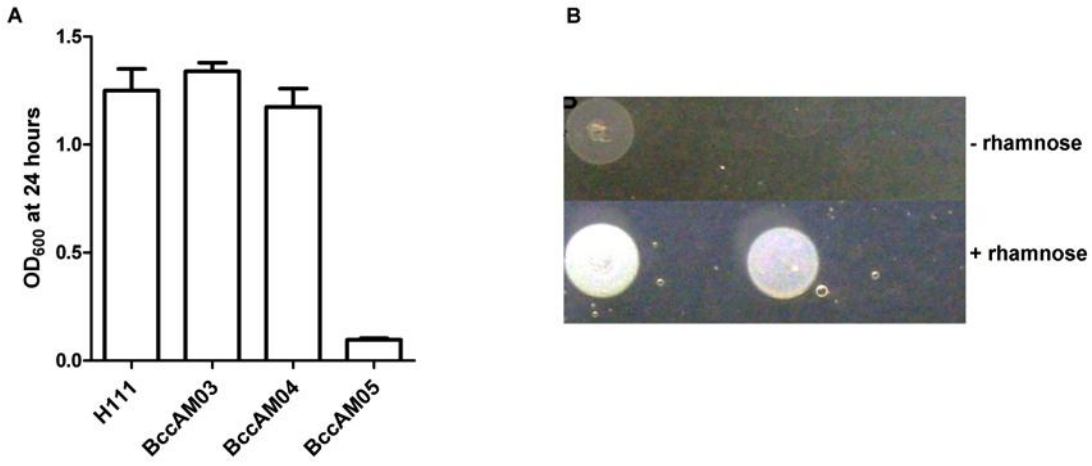


Figure 5: Role of *ftr*$_{Bcc}$*ABCD* in the growth and iron uptake of *B. cenocepacia* H111 in iron-restricted conditions. (A) Growth of H111, BccAM03, BccAM04 and BccAM05 in IFS medium after 24 hours of incubation. (B) Ten µL drops of serial dilutions of exponentially growing cultures of BccAM05 plated on to IFS agar with and without rhamnose. From left to right: suspensions of BccAM05 cultures diluted to OD_{600} = 0.5, 0.05 and 0.005. Induction of *ftr*$_{Bcc}$*ABCD* expression by rhamnose is sufficient to restore growth of BccAM05 in iron-restricted medium.

3.3.4 Ftr$_{Bcc}$ABCD is not required for uptake of ferrous iron in Bcc H111

The *ftrA* mutants of *Bordetella* and *Brucella* display strong growth defects when ferrous iron is the dominant iron species in the medium, for example at acidic pH and in the presence of the reducing agent ascorbate. This behavior was taken as evidence that the Ftr system is involved in uptake of ferrous iron (Brickman and Armstrong, 2012; Elhassanny *et al*., 2013). To test if the *ftr*$_{Bcc}$*C* mutant shows a similar defect when high-affinity uptake of ferrous iron is essential for optimal growth, we compared the growth of the H111 wild-type and the BccAM04 strain at pH 6 in the IFS medium supplemented with ascorbate and a wide range of $FeSO_4$ concentrations. These conditions ensure that virtually all the iron in the medium is in the ferrous form (Brickman and Armstrong, 2012). In addition, we supplemented the medium with desferoxamine to chelate ferric iron. Supplementation of IFS medium with 10µM desferoxamine could not rescue the growth of strain BccAM05, ruling out a possible role of desferoxamine as an exogenous siderophore (data not shown).

Under these conditions, we could not detect significant differences in the growth of the *ftr$_{Bcc}$C* mutant BccAM04 and the wild-type strain H111 (Figure 6 and Figure S3), in contrast to the results obtained with Ftr mutants in *B. pertussis* or *B. abortus* (Brickman and Armstrong, 2012 ; Elhassanny *et al.*, 2013). These data suggest that (1) *B. cenocepacia* H111 can acquire iron in its Fe^{2+} form independently of the Ftr_{Bcc} system and (2) the contribution of the Ftr_{Bcc} system to Fe^{2+} uptake in H111 is negligible.

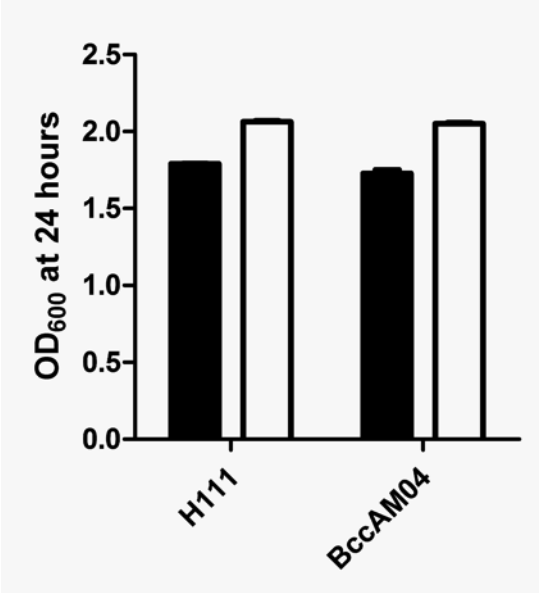


Figure 6: Growth of *B. cenocepacia* H111 and BccAM04 in IFS medium supplemented with ferric or ferrous iron. Cultures were grown at 37°C in IFS medium supplemented with 10 µM $FeCl_3$ at pH 7 (black bars) or in IFS medium buffered at pH 6 supplemented with 10 µM FeSO4, 2 mM ascorbate and 10 µM Desferoxamine (white bars). The optical density of the cultures was measured after 24h incubation. Error bars represent the standard error of three independent experiments

3.3.5 Ftr_{Bcc}ABCD is not involved in the uptake of complex iron substrates such as, hemin and lactoferrin

To test if the H111 Ftr_{Bcc}ABCD system is involved in scavenging iron from different sources, we measured the ability of the BccAM03, BccAM04, BccAM05 and wild-type H111 strains to utilize hemin, lactoferrin, hematite and ferritin. As shown in Figure

3.5, all strains tested could grow optimally with hemin as an iron source, suggesting that neither siderophores nor the Ftr_{Bcc}ABCD system were required for heme utilization. Supplementation of IFS medium with 10 M lactoferrin did not promote growth of strains BccAM03 and BccAM05. This shows that siderophores but not the Ftr_{Bcc} ABCD system, are required for the ability of H111 to utilize lactoferrin as substrate. In contrast, uptake of iron from ferric chloride or ferric oxide could proceed via either the siderophores or the Ftr_{Bcc} systems (Figure 7). A similar pattern was observed with utilization of ferritin: only the triple mutant BccAM05 was unable to grow normally with ferritin as a substrate. Extracellular proteases produced by *B. cenocepacia* have been shown to degrade ferritin *in vitro,* leading to release of mineral iron into the medium (Whitby *et al.*, 2006). Together, these findings suggest that the Ftr_{Bcc}ABCD system is an alternative mineral iron uptake system and is not specialized in scavenging iron from organic sources.

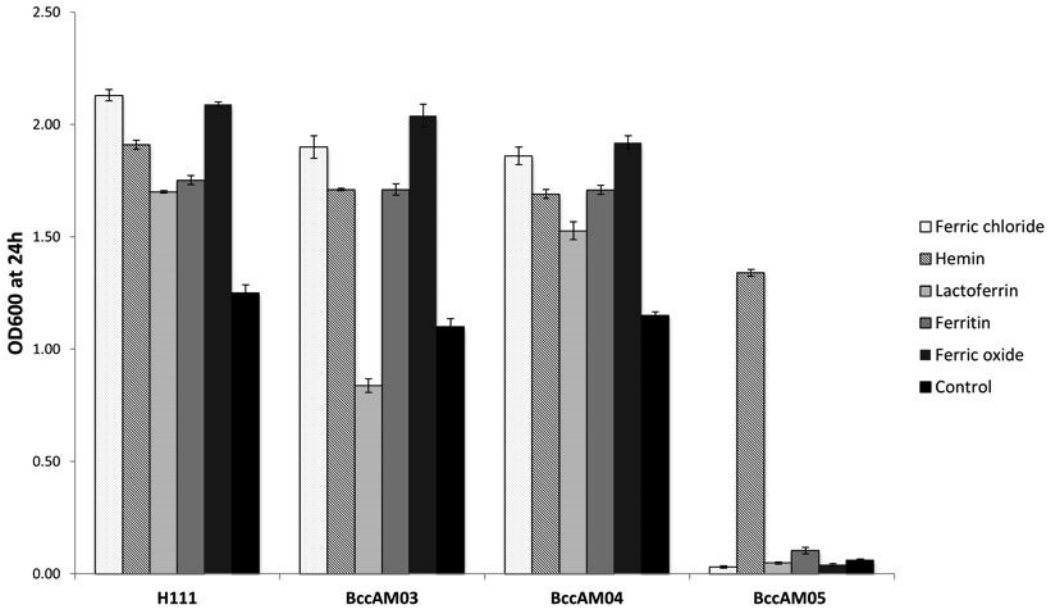


Figure 7: Substrate utilization by H111 wildtype and mutant strains. **Bacteria were cultured in IFS medium supplemented with various iron sources at 37°C. OD_{600} was measured after 24h of incubation. The concentrations of iron sources used were: ferric chloride, hemin, lactoferrin: 10 µM; Ferric oxide: 0.2mg/mL; Ferritin: 1 g/mL; Control: IFS medium without supplemented iron. Values are the average of three independents ± standard error.**

3.3.6 Ftr_{Bcc}ABCD is a high affinity iron transporter

To directly test whether Ftr_{Bcc}ABCD transports ferric iron, we measured the uptake of radiolabeled $^{59}FeCl_3$ by the H111 wild type and mutants BccAM03, BccAM04 and BccAM05. The siderophore mutant BccAM03 and the Ftr_{Bcc} mutant BccAM04 showed a slower iron uptake compared to the wild-type, indicating that both the siderophore and the Ftr_{Bcc} system contribute to iron uptake at low concentrations (1 □M $FeCl_3$). However, the triple mutant BccAM05 displayed a severely reduced uptake of ferric iron, suggesting that the Ftr_{Bcc}ABCD system is a high-affinity ferric iron uptake system (Figure 8). To confirm the species of iron preferred by the Ftr_{Bcc} system, we compared the uptake rate of $^{59}FeCl_3$ and $^{59}FeSO_4$ by BccAM03 and BccAM05. In all conditions and strains tested, uptake of ferrous iron was slower than uptake of ferric iron, indicating that *B. cenocepacia* H111 takes up ferric iron more efficiently than ferrous iron, either *via* siderophores or the Ftr_{Bcc} system. Uptake of both ferrous and ferric iron was reduced in strain BccAM05 compared to BccAM03 (Figure 8B and Figure 8C). Together, these data suggest that the Ftr_{Bcc} system can transport both ferrous and ferric iron. Our results also indicate that that uptake of ferric iron by the Ftr_{Bcc} system is about 50% faster than for ferrous iron.

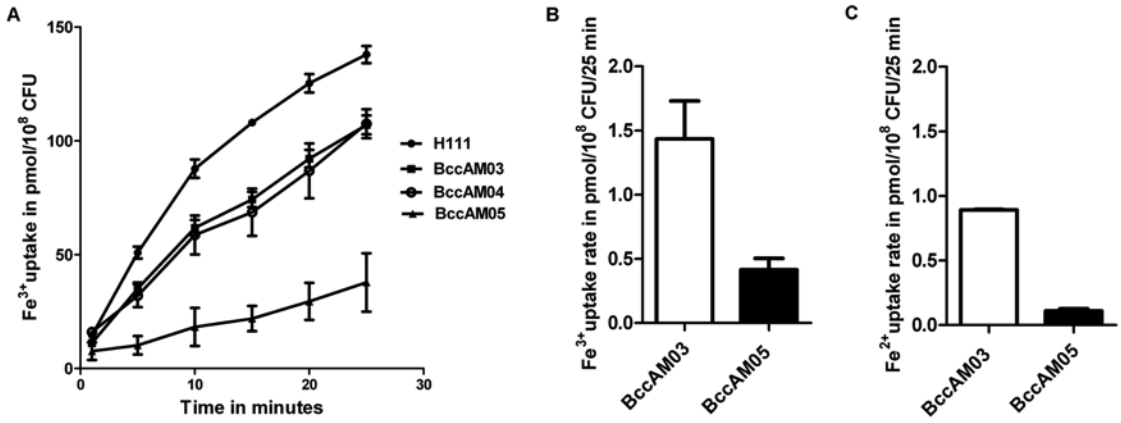


Figure 8: Kinetics of iron uptake by *B. cenocepacia ftr$_{Bcc}$ABCD* and siderophore mutants (A) $^{59}FeCl_3$ uptake by H111 (black circles), BccAM03 (black squares), BccAM04 (white circles) and BccAM05 (black triangles). Uptake assays were initiated by adding 1 µM $^{59}FeCl_3$ to the cultures (See Material and methods). Error bars represent the standard error of three independent experiments. Rate of uptake of $^{59}Fe^{3+}$ (B) and $^{59}Fe^{2+}$ (C) by BccAM03 and BccAM05 over a period of 25 minutes. 100 nM

$^{59}FeCl_3$ and 100 nM $^{59}FeSO_4$ were used for the uptake assay, respectively. 2 mM sodium ascorbate was included in the medium to keep $^{59}FeSO_4$ in the reduced form. Error bars represent the standard error of three independent experiments.

3.3.7 Heterologous expression of ftr_{Bcc}ABCD rescues the growth of an *E. coli* strain lacking all known high affinity iron uptake systems

In order to study Ftr_{Bcc}ABCD without interference by uncharacterized iron uptake systems of *Burkholderia*, we expressed the *ftr_{Bcc}ABCD* gene cluster from plasmid pAUM2 in *E. coli* GR536, a strain unable to grow in iron-limited medium because of a lack of all known iron uptake systems (Grass *et al*., 2005). Expression of *ftr_{Bcc}ABCD* from plasmid pAUM2 allowed the strain to grow well under iron limited conditions (Figure 9), indicating that heterologous expression of *ftr_{Bcc}ABCD* is sufficient to rescue the lack of a native high affinity iron transport system in that strain. *E. coli* GR536 (pAUM2) exhibited a significantly higher uptake of radiolabeled ferric iron compared to ferrous iron, substantiating the ferric iron preference of the Ftr_{Bcc} system, consistent with our observations in H111 (Figure 10A). We observed a similar behavior across a range of iron concentrations from 100 nM to 10 µM (data not shown). We next examined the kinetics of iron transport by Ftr_{Bcc}ABCD to determine if the system possesses the characteristics of a high-affinity ferric iron transporter. We measured $FeCl_3$ uptake kinetics of *E. coli* GR536 (pAUM2) grown in IFS medium (Figure 10B). Values of the kinetic parameters, Michaelis-Menten constant (Km) and maximum uptake (Vmax) are shown in Table 2. *E. coli* GR536 (pAUM2), lacking all major iron uptake systems but harboring a functional Ftr_{Bcc}ABCD system, displayed an apparent Km value of 1.6 µM, indicating that the Ftr_{Bcc} system has a high affinity for $FeCl_3$. This measure of affinity correlated with the kinetic parameters obtained for the H111 strain deficient in siderophores but possessing the Ftr_{Bcc}ABCD system (Table 5). We could not accurately estimate the values of kinetics parameters for the uptake of ferrous iron by *E. coli* GR536 (pAUM2) because Fe^{2+} transport had not reached its maximum rate at the highest concentration tested (10 M). We can only conclude that the Km value of Ftr_{Bcc} system for ferrous iron is greater than 5 µM. These data strongly support the hypothesis that the H111 Ftr_{Bcc}ABCD transport system is specific for ferric iron.

Addition of 10 µM of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) or incubation at 4°C completely inhibited iron uptake by BccAM03, indicating that iron transport by $Ftr_{Bcc}ABCD$ requires metabolically active cells (Figure S2). This result rules out the possibility of passive binding of iron to the bacterial cell surface.

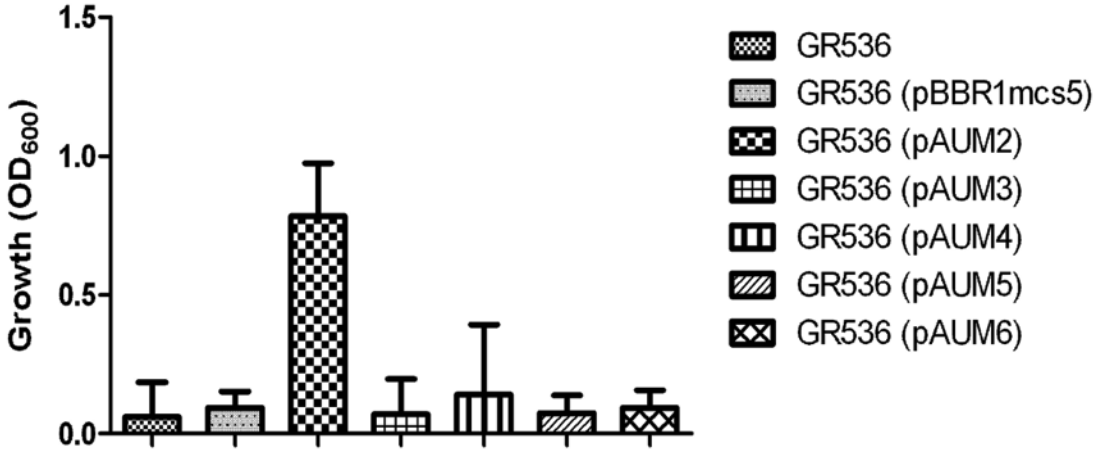


Figure 9: Growth of *E. coli* GR536 complemented with all or parts of the *$ftr_{Bcc}ABCD$* operon. Plasmid pAUM2 harbors all the components of the *$ftr_{Bcc}ABCD$* operon while its derivatives contain targeted non-polar deletions of individual *ftr_{Bcc}* genes (see Results section for details). Cultures were incubated at 37°C in IFS medium and OD_{600} was measured after 48h. Data represent the mean ± standard error of three independent experiments.

3.3.8 All four components of $Ftr_{Bcc}ABCD$ are essential for iron uptake

We next sought to determine which components of $Ftr_{Bcc}ABCD$ were essential for high affinity iron uptake. We therefore evaluated the ability of single deletion mutants of *$ftr_{Bcc}ABCD$* to rescue growth of *E. coli* GR536. Nonpolar deletion of any single component of this system resulted in a complete loss of growth in iron-limited medium (Figure 9). This shows that all four proteins encoded by the operon are necessary for iron transport as has been reported for the Ftr system in *B. pertussis* (Brickman and Armstrong, 2012).

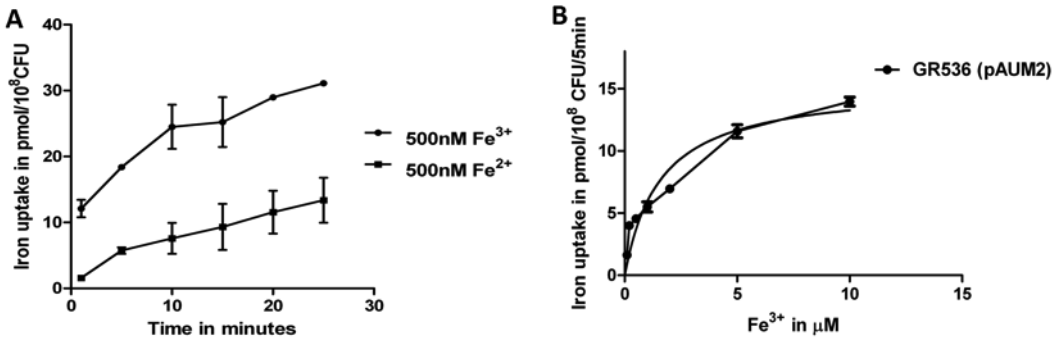


Figure 10: Kinetics of iron uptake by *E. coli* GR536 harboring *ftr$_{Bcc}$ABCD* (A) Net Uptake of 500 nM ferric (circles) and 500 nM ferrous (squares) iron by *E. coli* GR536 expressing Ftr$_{Bcc}$ABCD. Residual iron uptake rates of the parental strain *E. coli* GR536 was subtracted to obtain the net iron uptake rate attributable to Ftr$_{Bcc}$ABCD. Data represent the mean ± S.E of three independent experiments (B) Initial velocity of $^{59}FeCl_3$ transport by *E. coli* GR536 (*ftr$_{Bcc}$ABCD*) vs. $FeCl_3$ concentration. The initial iron uptake rate was determined from the iron uptake slope over a concentration range of 100 nM – 10 µM of $^{59}FeCl_3$ at 37°C as described in the Material and Methods. Regression (smooth line) was used to estimate the values of the Michaelis- Menten constant (Km) and maximum uptake rate (Vmax) (Table 1). Data represent the mean ± S.E of three independent experiments

Table 2: Kinetic parameters of iron uptake by *E. coli* GR536 (*ftr$_{Bcc}$ABCD*)

Strain	Km (µM)	Vmax (pmol/10^8CFU/5min)
GR536 (*ftr$_{Bcc}$ABCD*)	1.6 ± 0.39	15.37 ± 1.28
BccAM03	1.1 ± 0.52	47.23 ± 1.92

Bacteria grown in IFS medium were washed and resuspended in IFS medium supplemented with radiolabeled $FeCl_3$. Vmax and Km values were calculated obtained as described in Material and Methods. Values given are averages of three independent experiments ± standard error.

3.3.9 Transcription of *ftr$_{Bcc}$ABCD* is regulated by iron limitation, not by pH

The Ftr systems of *B. pertussis* and *B. abortus* were shown to be highly up-regulated at low pH as well as under low iron conditions, consistent with a role of the Ftr system in the uptake of ferrous iron in these organisms (Brickman and Armstrong, 2012; Elhassanny *et al*., 2013). We speculated that since the apparent physiological role of Ftr$_{Bcc}$ABCD is iron (III) uptake, regulation of the *ftr$_{Bcc}$ABCD* operon might follow different rules.

Using real time PCR we determined that transcription of *ftr$_{Bcc}$ABCD* was highly induced when H111 was grown under iron restricted IFS medium compared to growth in IFS medium supplemented with 50 □M of $FeCl_3$ (Table 3). This is similar to the regulation of the *ftr* genes of *B. abortus* and B*. pertussis*. Transcription of the *ftr$_{Bcc}$ABCD* genes was only slightly induced (2-fold increase) when bacteria were grown in IFS medium at pH 5 compared to pH 7 (Table 2). This is in stark contrast to the expression of the *ftr* system in *B. pertussis* and *B. abortus,* which were increased 8- and 24-fold, respectively, when grown at pH 6 relative to pH 7 (Brickman and Armstrong, 2012; Elhassanny *et al*., 2013). Our finding correlates with the regulation pattern observed for the related *Burkholderia* strain *B. cenocepacia* J2315 at low pH (Sass *et al*., 2013). Taken together, these observations indicate that the *Burkholderia* Ftr$_{Bcc}$ system is strongly induced by iron limitation, but not by acidic pH.

Table 3: Relative expression levels of ftr$_{Bcc}$A

Conditions	Fold change	P value
Iron restricted Vs. iron replete[a]	**20.6 ± 0.596**	**0.0001**
pH5 Vs. pH7[b]	**2.106 ± 0.1815**	**0.0100**

Cultures were harvested at mid-exponential phase, and expression of *ftr$_{Bcc}$A* was measured by quantitative RT-PCR (See Material and Methods). Values given are the average of three independent experiments ± standard deviation. Student T-test statistic values are given (p-value).
a. Bacteria were cultured at 37°C in IFS medium at pH7 supplemented with 60 µM Dipyridyl (iron restricted); 50 µM $Fecl_3$ (iron replete).

3.3.10 The role of Ftr_{Bcc}ABCD for virulence in *Galleria mellonella* infection model

Iron uptake *via* siderophores is an important factor for host invasion by *Bcc* bacteria and disease progression (Sokol *et al*., 2000; Visser *et al*., 2004). We tested whether uptake of iron through Ftr_{Bcc}ABCD plays a role in pathogenicity by analyzing the virulence of H111, the siderophore mutants BccAM01, BccAM02 and BccAM03 and the *ftr_{Bcc}ABCD* mutants BccAM04 and BccAM05 in the *G. mellonella* infection model. We did not observe any significant difference in survival of *G. mellonella* when the larvae were infected with the wild type H111 or the pyochelin-deficient mutant BccAM01 (Figure 11B and 11C). However, both the ornibactin-deficient mutant BccAM02 and strain BccAM03, deficient in production of both pyochelin and ornibactin, were attenuated, suggesting that ornibactin but not pyochelin is essential for virulence (Figure 11D and 11E). The *ftr_{Bcc}C* mutant BccAM04 showed wild type levels of virulence, demonstrating that functional siderophore systems can compensate for a defective Ftr_{Bcc} system *in vivo* (Figure 11G). Finally, *G. mellonella* larvae infected with BccAM05 displayed the highest survival rates, undistinguishable from mock-infected larvae (Figure 11H). These results show that: (1) ornibactin biosynthesis is critical for virulence, while pyochelin production plays a subordinate role; and (2) Ftr_{Bcc}ABCD-dependent iron uptake plays a minor, discernible role in pathogenesis only when siderophores are absent.

3.3.11 Distribution of Ftr_{Bcc}ABCD in *Burkholderia* species

Given that the Ftr_{Bcc} system is not important for persistence during infection we speculated that it might be important for iron uptake of *Burkholderia* sp. in the environment. We searched for the *ftr_{Bcc}ABCD* gene cluster in all published *Burkholderia* sp. genomes, with lifestyles ranging from animal pathogens to soil isolates and plant beneficial bacteria, to investigate whether the system is associated with a particular lifestyle or niche. Analysis of 59 publicly available *Burkholderia* sp. genome sequences, including incomplete draft sequences, showed that all but one encode the Ftr_{Bcc}ABCD system. Closer analysis of the *Burkholderia mallei* 2002721280 draft genome sequence, the only one negative for the system, showed that the gene cluster is almost complete, with the exception of the putative ortholog

of *ftr$_{Bcc}$C,* which is a pseudogene. A list of putative orthologs and their respective positions are listed in the supplementary table (Table S3). Ftr$_{Bcc}$ABCD is therefore ubiquitous in *Burkholderia* species, even in obligate symbionts of fungi and plants such as *B. rhizoxinica* and *Candidatus* B. kirkii. In addition, screening of several *Burkholderia* isolates on CAS plates revealed that some environmental isolates do not produce siderophores (Table S4). Nevertheless, PCR amplification performed on four of these isolates detected the presence of the *ftr$_{Bcc}$ABCD* genes (data not shown). The Ftr$_{Bcc}$ system is therefore a common feature of *Burkholderia* species, while the presence of siderophores seems facultative.

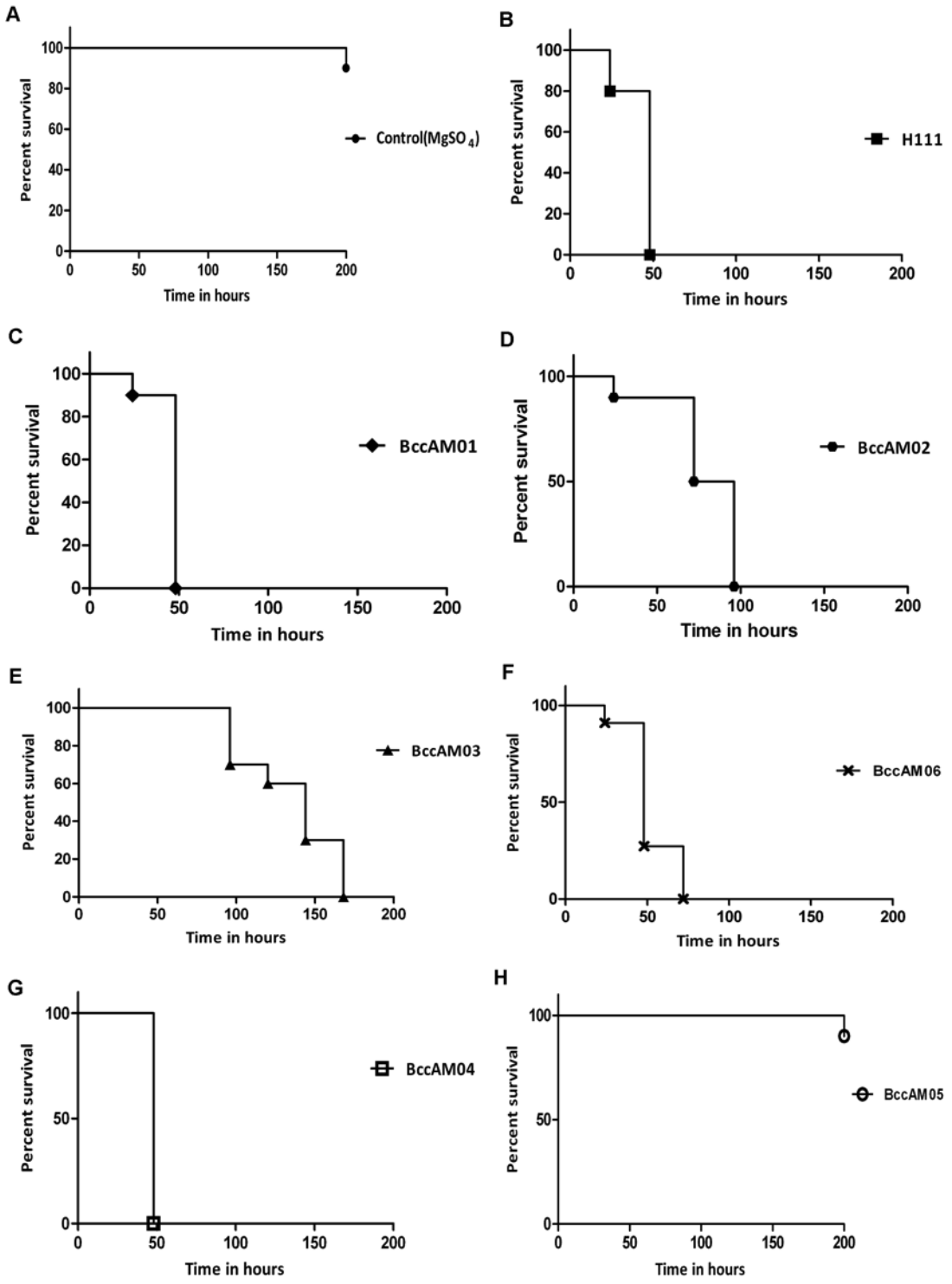


Figure 11: Conribution of siderophores and the Ftr_{Bcc}ABCD system to the virulence of H111. Kaplan-Meier plot of *G. mellonella* survival after infection with bacterial suspensions of (A) $MgSO_4$ (mock) (B) H111 (C) BccAM01 (D) BccAM02 (E) BccAM03 (F) BccAM06 (G) BccAM04 and (H) BccAM05. Strains BccAM02 and BccAM03 were significantly less virulent than the wild-type H111 (Log rank test P-value < 0.05). Strains BccAM01, BccAM04 and BccAM06 were not significantly less virulent than H111. Finally, strain BccAM05 was completely avirulent, indistinguishable from the mock infection (P-value > 0.05).

3.4 Discussion

Siderophore-mediated transport is generally considered to be the primary means of iron uptake and therefore a major virulence factor in many pathogenic bacterial species including *Burkholderia* sp. (Ratledge and Dover, 2000; Uehlinger *et al*., 2009). Members of the Bcc are known to produce up to four siderophores, namely pyochelin, ornibactin, cepabactin and cepaciachelin (Thomas, 2007). Pyochelin and ornibactin are produced by our model strain *B. cenocepacia* H111. Ornibactin was previously shown to compensate for a non-functional pyochelin system during infection in the closely related strain *B. cenocepacia* Pc715j (Visser *et al*., 2004). However, the effect of a complete absence of siderophores on growth and lifestyle of *Burkholderia* sp. has not been investigated. When the two siderophore systems present in our model strain *B. cenocepacia* H111 were inactivated, the resulting mutant strain, BccAM03, did not show any growth defect, even when cultured under iron limitation (figure 1).

We noticed that the H111 genome encodes a putative iron transport system, which we named Ftr_{Bcc}ABCD, that is homologous to the recently described FtrABCD systems of *B. pertussis* (Brickman and Armstrong, 2012) and *B. abortus* (Elhassanny *et al*., 2013), which were shown to be involved In the uptake of ferrous iron in these organisms (Figure 2). The $ftr_{Bcc}C$ gene, coding for the putative iron permease of the Ftr_{Bcc}ABCD system belongs to the iron/lead transporter (ILT) superfamily (Transporter classification database entry 9.A.10) (Debut *et al*., 2006). Members of this group have been identified and associated with iron uptake in *S. cerevisiae* (FTR1) (Stearman *et al*., 1996), *E. coli* (EfeU and FetM) (Grosse *et al*., 2006; Cao *et al*., 2007; Koch *et al*., 2011) , *B. pertussis* (FtrC) (Brickman and Armstrong, 2012) and *B. abortus* (FtrC) (Elhassanny *et al*., 2013). In addition, some components of the Ftr_{Bcc}ABCD system share structural domains of the Ftr1/Fet3 system of *S. cerevisiae* (Stearman *et al*., 1996) and the EfeUOB (Grosse *et al*., 2006; Cao *et al*., 2007) and FetMP systems of *E. coli* (Koch *et al*., 2011).

Recent studies in *B. abortus* and *B. pertussis* have shown that the FtrABCD systems are ferrous iron uptake systems, as *ftr* mutants failed to grow in media with Fe^{2+} as sole iron source (Brickman and Armstrong, 2012; Elhassanny *et al*., 2013). In

contrast, the *B. cenocepacia* H111 mutant BccAM04, which harbors a defective Ftr_{Bcc}ABCD system, displayed robust growth in a medium containing $FeSO_4$, reagents aiding stability of Fe^{2+} and a specific Fe^{3+} chelator (Figure 6 and S3). We conclude from these experiments that *B. cenocepacia* H111 uses the Ftr_{Bcc}ABCD system to import ferric iron and that the genome of *B. cenocepacia* H111 encodes an as yet unidentified ferrous iron uptake transporter.

Consistent with a role in ferric vs. ferrous iron uptake, we show that transcription of the *ftr_{Bcc}ABCD* genes is up-regulated when iron is limited, but not in response to low pH, at which ferrous iron is more stable in solution (Table 3, Morgan and Lahav, 2007). This is in contrast to the *ftrABCD* genes of *B. abortus* sp. and *B. pertussis,* which are induced in response to both iron limitation and low pH (Brickman and Armstrong, 2012; Elhassanny *et al*., 2013).

In order to study uptake of iron by Ftr_{Bcc}ABCD without interference by uncharacterized iron uptake systems, we expressed the *ftr_{Bcc}ABCD* genes *in trans* in *E. coli* strain GR536, lacking all known high-affinity iron transport systems (Grass *et al*., 2005). Under our experimental conditions, expression of *ftr_{Bcc}ABCD* was sufficient to restore growth of *E. coli* GR536 in iron-limited media (Figure 9). The observed Km value of 1.6 µM for Fe^{3+} in *E. coli* GR536 pAUM2 indicates that Ftr_{Bcc}ABCD is a high affinity transport system for ferric iron, with a Michaelis constant on par with that of the siderophore-dependent systems of *B. cenocepacia* H111 and other species (data not shown; Crowley *et al*., 1991). Based on these observations, we believe that the Ftr_{Bcc}ABCD functions as an alternative siderophore-independent, high-affinity ferric iron uptake system in the genus *Burkholderia*.

The most compelling argument that Ftr_{Bcc}ABCD is a high-affinity ferric iron uptake system comes from our $^{59}FeCl_3$ uptake assays. Heterologous expression of the *ftr_{Bcc}ABCD* genes in *E. coli* GR536 enabled the mutant strain to take up Fe^{3+} at a significantly faster rate than Fe^{2+} (Figure 10). However, both ferric and ferrous iron uptake were found to be reduced in the H111 triple mutant BccAM05 relative to the siderophore null mutant BccAM03 (Figure 8). A possible explanation is that Fe^{2+} serves as an intermediate in the transport of Fe^{3+} by the Ftr_{Bcc} system. Alternatively,

it may be that the Ftr_{Bcc} system allows ferrous iron transporters to function more efficiently in *B. cenocepacia* H111. As these alternative ferrous transporters are absent in *E. coli*, the indirect effect of the *ftr_{Bcc}ABCD* genes on ferrous iron uptake would be lost. This situation is reminiscent of the study of Mey and coworkers, who showed that mutations in the *vciB* gene, encoding a putative TonB-dependent receptor, inhibited the activity of native ferrous iron transporters *via* an indirect mechanism in *Vibrio cholerae* (Mey *et al*., 2008). Both explanations are consistent with the fact that the Ftr_{Bcc} system is indispensable for growth on ferric iron when siderophores are absent, even at relatively high concentrations (Figure 5). In contrast, the growth of an Ftr_{Bcc}ABCD mutant and the wild-type are indistinguishable when ferrous iron is present in the culture medium at 10 M (Figure 4). We conclude that the physiological role of the Ftr_{Bcc}ABCD system in *B. cenocepacia* is transport of iron (III) rather than iron(II). *B. cenocepacia* H111 is a strict aerobe and, contrary to the pathogens *B. abortus* and *B. pertussis*, does not replicate intracellularly in the host (Pessi *et al*. 2013; Lamothe *et al*. 2006). It could therefore be argued that *B. cenocepacia* encounters iron mostly in ferric form or bound to complex substrates like hemin, ferritin or lactoferrin in the host.

We demonstrated that the Ftr_{Bcc}ABCD system allows H111 to effectively acquire iron from iron oxide particles (hematite). We speculate that organic acids (e.g. gluconate), secreted by growing *Burkholderia* species, including H111, may play a role in the solubilization of iron oxide under oxic, neutral pH conditions (Delvasto *et al*., 2006; Henry *et al*., 2001). We hypothesize that ferric iron-organic acid complexes can enter the periplasm through porins where it is subsequently reduced by the putative periplasmic P19 protein Ftr_{Bcc}A. Ferrous iron is then translocated across the cytoplasmic membrane by Ftr_{Bcc}C. The polyferredoxin protein Ftr_{Bcc}D and the cupredoxin Ftr_{Bcc}B are possibly responsible for shuttling electrons during the transport process across the cytoplasmic membrane.

In contrast to the Ftr system in *B. abortus*, the Ftr_{Bcc} system appears to be dispensable for virulence of H111 (Figure 11, Elhassanny *et al*., 2013). In agreement with previous studies we demonstrated the importance of siderophores for the pathogenicity of *B. cenocepacia* (Sokol *et al*., 1999; Visser *et al*., 2004; Uehlinger *et*

al., 2009). Interestingly, siderophores were shown to be irrelevant in establishing infection by *B. abortus* (Bellaire *et al*., 1999; Gonzalez carrero *et al*., 2002). This disparity in the role of iron uptake pathways in virulence could be attributed to different lifestyles, as described for several fungal species possessing both siderophores and reductive iron assimilation mechanisms (Schrettl *et al*., 2004; Johnson *et al*., 2013). Elhassanny *et al*. proposed that *B. abortus* utilizes the Ftr system to acquire iron from its ferrous form in the host's intracellular compartments, thus explaining the critical role of the Ftr system in virulence (Elhassanny *et al*., 2013). *B. cenocepacia* colonizes the lungs of CF patients where iron is predominantly sequestered by transferrin, lactoferrin, ferritin and hemin (Whitby *et al*., 2006). Siderophores and proteases may therefore be crucial for acquiring iron from such extracellular sources. We demonstrated that *B. cenocepacia* could rely on either the Ftr_{Bcc}ABCD system or siderophores to scavenge iron from ferritin, while siderophores were critical for utilization of lactoferrin (Figure 5). Our data also show that H111 can acquire iron from hemin independently of siderophores and the Ftr_{Bcc}ABCD system, pointing to the presence of additional transporters that are likely necessary for the virulence of this organism.

In order to establish a link between lifestyle and the Ftr_{Bcc} system, we examined the production of siderophores and the prevalence of the $ftr_{Bcc}ABCD$ gene cluster across the genus *Burkholderia* (Table S3). A recent study suggested that the genus *Burkholderia* may be divided into two main clades: one containing pathogenic *Burkholderia* species, including the BCC, the *pseudomallei* lineage and plant pathogens, and the other consisting of non-pathogenic *Burkholderia* species that are mostly associated with plants (Suárez-Moreno *et al*., 2012). We found that the $ftr_{Bcc}ABCD$ genes were present in all *Burkholderia* species whose genomes were available at the time of our study whereas some members of the non-pathogenic *Burkholderia* cluster did not synthesize siderophores. This suggests that Ftr_{Bcc}ABCD is a common iron uptake strategy in *Burkholderia* species while siderophores appear to be particularly important for pathogenic strains. The Ftr_{Bcc} system might be particularly advantageous under conditions where siderophores are ineffective, for example when diffusion in the environment is not limited. Furthermore,

siderophores such as pyoverdine can become permanently adsorbed at the surface of metal oxide particles (Upritchard *et al*., 2007). Hence, Ftr_{Bcc}ABCD-dependent uptake might be a less wasteful iron acquisition strategy for example in soil, which not only contains high amounts of metal oxides but is also the natural habitat of many *Burkholderia* species. Additional work is required to test whether the different iron uptake systems can provide a competitive advantage in a particular habitat.

In conclusion, we provide evidence that the Ftr_{Bcc} system of *Burkholderia* sp. and the Ftr systems of *Brucella* and *Bordetella* species represent a new class of polyvalent iron transporters in bacteria. These polyvalent transporters are capable of transporting either Fe^{2+} or Fe^{3+}, although the molecular basis for specificity of the two systems remains to be elucidated. The ion specificities of the two systems are in agreement with their different expression patterns and the environmental niches of their hosts: While the Ftr systems of *Brucella* and *Bordetella* are induced at low pH and are required for the uptake of iron (II), possibly during infection, the Ftr_{Bcc} system of *B. cenocepacia* is not induced at low pH but under iron limiting conditions, is a Fe(III) uptake system and is not essential for pathogenicity.

3.5 Materials and Methods

3.5.1.Bacterial strains and media

Strains and plasmids used in this study are listed in Table S1 and primers are listed in Table S2. *Burkholderia cenocepacia* H111, an isolate from a cystic fibrosis patient in Germany (Gotschlich *et al*., 2001) was the model strain used in this study. *Burkholderia* strains were grown at 37^{o}C in iron limited succinate medium (IFS) (Meyer and Abdallah, 1978) for all experiments unless otherwise indicated. Potassium phosphate buffer (15mM) was used to adjust the IFS medium to pH7 unless otherwise indicated. IFS was buffered at pH 6 by 25 mM 3-(N-morpholino)propane sulphonic acid (MOPS) and at pH5 by adjusting the proportion of phosphate salts to (in g/L): 1.65 KH_2PO_4, 0.35 NaH_2PO_4, 0.15 K_2HPO_4 x 3 H_2O, 0.017 Na_2HPO_4 (Sass *et al*., 2013) .

IFS medium was supplemented with thiamine (10µM) for growth of *E. coli* strains. In some experiments, traces of iron were removed from the medium by complexing with chelators such as 2, 2'-dipyridyl (Sigma) or desferoxamine (Sigma). Glassware was rinsed with 2.4 N HCl followed by deionized water to remove any traces of iron. When required, antibiotics were added at the following concentrations; 50 µg ml^{-1} of trimethoprim, 20 µg ml^{-1} of gentamicin, 20 µg ml^{-1} of chloramphenicol, 50 µg ml^{-1} of kanamycin and 100 µg ml^{-1} of ampicillin.

E.coli strains Top 10 (Invitrogen) and cc118λpir (Invitrogen) were used as cloning hosts. *E. coli* strains were grown in Luria Bertani (LB) medium at 37°C unless otherwise mentioned.

3.5.2.Bacterial genetics

Pyochelin and ornibactin mutant strains of *B. cenocepacia* H111 were constructed as previously described (Carlier *et al*., 2009). Flanking regions of *pchAB* and *orbJ* were amplified by PCR using oligonucleotide primers listed in Table S2. A kanamycin resistance cassette from plasmid pKD4 (Datsenko and Wanner, 2000) was inserted between the flanking regions of *pchAB* and *orbJ* using an overlap PCR reaction. The resulting PCR fragments were cloned into the Gateway Entry vector pDONR221 using the BP clonase II kit (Invitrogen, Carlsbad, CA, USA). The constructs were then transferred into the suicide vector pAUC40, a Gateway-compatible derivative of the plasmid pKNG101 (Carlier *et al*., 2009), using LR clonase II kit (Invitrogen, Carlsbad, CA, USA). The resulting plasmids were transferred to *B. cenocepacia* H111 by triparental mating and allelic replacement selected for kanamycin resistance. Clones were screened for resistance to sucrose, denoting excision of the *sacB*-harboring pAUC40 plasmid backbone. Double cross-over allelic replacement events were further verified by PCR. Markerless deletion mutants BccAM01 (H111 *ΔpchAB*) , BccAM02 (H111 *ΔorbJ*) and BccAM03 (H111 *ΔpchABΔorbJ*)] were then generated by excising the kanamycin resistance cassette using Flp recombinase from pFLP2 (Choi and Schweizer, 2005; Agnoli *et al*., 2012). For complementation of the mutants, *pchAB* and *orbJ* genes and their flanking regions containing the predicted native promoters were amplified by PCR using primers listed in table S2 and cloned into the

vector pBBR1MCS-5. The resulting plasmids were introduced into *E.coli* top 10 and transferred into BccAM03.

To generate rhamnose-dependent conditional mutants of *B. cenocepacia* H111, 300 bp fragment of $ftr_{Bcc}C$ starting from the start codon was amplified and ligated into the vector pSC200(Ortega *et al*., 2007). The resultant recombinant plasmid, in which expression of $ftr_{Bcc}C$ is controlled by a rhamnose-inducible promoter, was transformed into *E. coli* cc118λpir. It was subsequently transferred to the wild type H111 and the siderophore mutant BccAM03 by triparental mating. Homologous recombination resulted in insertion of a rhamnose-inducible promoter directly upstream of $ftr_{Bcc}C$. The conditional mutants, designated BccAM04 and BccAM05, respectively, were selected on Pseudomonas Isolation Agar (PIA) medium supplemented with Trimethoprim (100 µg ml^{-1}).

3.5.3.Sequence analysis

The draft H111 genome nucleotide sequence is publically available in the European Nucleotide Archive with the master WGS Accession No. CAFQ00000000. The clustal omega algorithm (http://www.ebi.ac.uk/Tools/msa/clustalo/), from EMBL-EBI, was used to perform multiple sequence alignment of different protein sequences. *E.coli* EfeU protein sequence (GI: 251784555) and Ftr1 sequence of *Saccharomyces cerevisiae* (GI: 256272065) were obtained from the NCBI protein database. TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM/) was employed for membrane topology predictions and homology searches of amino acid sequences in the GenBank and EMBL databases was carried out using the NCBI BLAST program. Interproscan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) enabled identification of conserved domains. Position of transcription starts was determined by mapping sequencing reads from publicly available RNAseq experiments (Pessi *et al*., 2013) in the CLC Genomics workbench (CLC bio, Aarhus, Denmark).

3.5.4.CAS assay

Bacteria were screened for the production of siderophores using Chrome Azurol S (CAS) assay described by Schwyn and Neilands (Schwyn and Neilands., 1987). Briefly, 5µl of exponential bacterial culture in iron limited IFS medium were spotted on CAS

agar plates, incubated at 37°C for 72 hours. Bacterial colonies were checked for the presence of discolored halo, which denotes siderophore production.

3.5.5.Growth assay and Iron source utilization test

B. cenocepacia H111 and AM03 were grown overnight in IFS medium while BccAM04 and BccAM05 were grown in IFS medium supplemented with 0.2% rhamnose. The cultures were pelleted, washed and resuspended in the IFS without any additives. The suspensions were incubated at 37°C for 30 minutes and serial dilutions of the strains were dropped on to IFS agar plates and growth monitored after 17 hours. To estimate the role of Ftr_{Bcc}ABCD in iron uptake at different pH, H111 wild type and different mutant strains were grown in IFS medium at pH 7 supplemented with 10 µM $FeCl_3$ and at pH 6 supplemented with 10 µM $FeSO_4$, 2 mM ascorbate and 10 µM desferoxamine.

To test whether *ftr_{Bcc}ABCD* is involved in scavenging iron from different iron sources, H111 wild-type and the mutant strains were grown in rich medium overnight. Absorbance of the cultures at 600nm was measured and aliquots normalized for cell density were used to inoculate IFS medium supplemented with 1 µg.ml^{-1} of equine ferritin (Sigma), 10 µM lactoferrin (Sigma), 10 µM hemin (Fluka) or 0.2 mg/mL of hematite (Sigma) and growth monitored after 24h. One, 10 and 50 µM ferric and ferrous sulfate (Sigma) were also used to determine the substrates of Ftr_{Bcc} system. Growth was monitored by measuring the optical density of the cultures at 600nm.

3.5.6.Iron uptake assay

The iron uptake assay was performed using radiolabeled $^{59}FeCl_3$ (Perkin Elmer Inc, US) as previously described (Cox, 1980; Sokol *et al*., 1999). $^{59}FeSO_4$ was prepared by reducing $^{59}FeCl_3$ using 2mM ascorbate in the presence of unlabelled $^{59}FeSO_4$. Bacteria were grown in IFS medium at pH 7 or pH 6 to mid-exponential phase, washed and resuspended in fresh medium at an OD_{600} of 0.3. Iron uptake assays in these cultures were initiated by the addition of 0.1 µCi of $^{59}FeCl_3$ or $^{59}FeSO_4$. At 10 min intervals, 1 ml samples of the culture were removed, placed on filters (pore size, 0.45 m; Millipore Corp.), and vacuum filtered. The filters were washed with 10 ml of 10mM Tris (pH 7.5)–0.9% NaCl and analyzed in a Packard liquid scintillation

counter (Packard Instrument Company, US). Values were normalized by deducting background values, which was obtained by performing the experiment with heat-killed cells.

To calculate the kinetic parameters of ferric iron uptake, the initial iron uptake rate was determined from the iron uptake slope over a concentration range of 100 nM to 10 µM of $^{59}FeCl_3$ at 37°C. The initial uptake rate was then plotted vs increasing concentrations of iron and the data fitted to the Michaelis-Menten equation using Prism software from Graphpad. Vmax and Km values are calculated using the same software. Residual iron uptake values of the host strain *E. coli* GR536 was also determined and deducted from the values obtained for GR536 harboring $Ftr_{Bcc}ABCD$.

3.5.7.Heterologous expression of $ftr_{Bcc}ABCD$

The *$ftr_{Bcc}ABCD$* gene cluster was amplified from H111 genomic DNA by PCR and cloned into the plasmid, pBBR1MCS-5 and the plasmid harboring the *$ftr_{Bcc}ABCD$* operon was then transformed into *E. coli* strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*) that lacks all major iron uptake systems (Grass *et al.*, 2005) .

To identify the essential components of the $Ftr_{Bcc}ABCD$ system, each of the genes was mutated as described previously (Erster and Liscovitch, 2010) with modifications. Primers pairs (listed in table S2) were used to amplify the entire plasmid pAUM2, excluding single genes of the *$ftr_{Bcc}ABCD$* operon, using a modified inverse PCR, resulting in plasmids pAUM3 to pAUM6. Amplification reactions resulted in linear plasmids, lacking single components of the operon. The resulting products were then subjected to DpnI degradation, which digests the methylated template plasmid while leaving only the PCR-amplified plasmid intact. The open plasmid was then circularized using T4 DNA ligase and transformed in to *E. coli* GR536.

3.5.8.Analysis of transcriptional regulation of $Ftr_{Bcc}ABCD$ by quantitative Real-Time qPCR

B. cenocepacia H111 cells were grown to mid-exponential phase in IFS medium supplemented with 50 µM $Fecl_3$ or 100 µM 2-2'dipyridyl. The pH of IFS medium was

adjusted to pH 5 or pH 7 by addition of sodium hydroxide before sterilization by filtering. The pH of the cultures was checked immediately prior to harvesting of the bacteria. RNA was extracted as previously described (Pessi *et al*., 2007) and further purified using RNeasy Qiagen kit. First strand cDNA was synthesized using random primers (Invitrogen) and MLV reverse transcriptase (Promega). qPCR was performed on the generated cDNA using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent, Switzerland) and a Mx3000P instrument (Agilent, Switzerland). Primers were generated using Primer 3 software(Rozen and Skaletsky, 1998) and are listed in Table S1. Each reaction was run in triplicates and melting curve data was analysed to determine the PCR specificity. The *recA* gene was used as the reference gene for normalization. Relative expression levels of the target gene, $ftr_{Bcc}A$, were calculated as previously described (Pfaffl, 2001). Ornibactin biosynthesis gene, *orbJ*, was used as a standard for evaluating H111 mRNA levels at iron replete and iron deplete conditions.

3.5.9.Galleria mellonella pathogenicity assay

Infection of *G. mellonella* larvae was performed as described previously (Seed and Dennis, 2008; Uehlinger *et al*., 2009). Briefly, *G. mellonella* in the final larval stage (purchased from Fischerei Brumann, Zürich) were stored in wood shavings at 15°C and used within 1 week. Overnight cultures of bacteria in LB broth were diluted 1:100 in 30 ml LB broth and incubated with shaking at 30°C to OD_{600} of 0.4–0.7. The bacteria were harvested by centrifugation and the pellets resuspended in 10 mM MgSO4 (Merck). Culture density was adjusted to an OD_{600} of 0.025, corresponding to 4 X 10^7 cfu ml^{-1}. Ten microliter aliquots were injected into the *G. mellonella* larvae via the hindmost proleg using a 1 ml syringe (BD Plastipak) with a 27-gauge by 7/8-in. needle (Rose GmbH, Germany). Mock infections were prepared by injecting 10 µl of $MgSO_4$ in the larvae. Ten larvae were used per strain tested, and each experiment was carried out in triplicate. The infected animals were incubated in Petri-dishes at 30°C in the dark. The number of dead larvae was counted every 24 hours for 7 days post infection. Larvae were considered dead when they did not respond to touch.

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3.6. Supplementary data

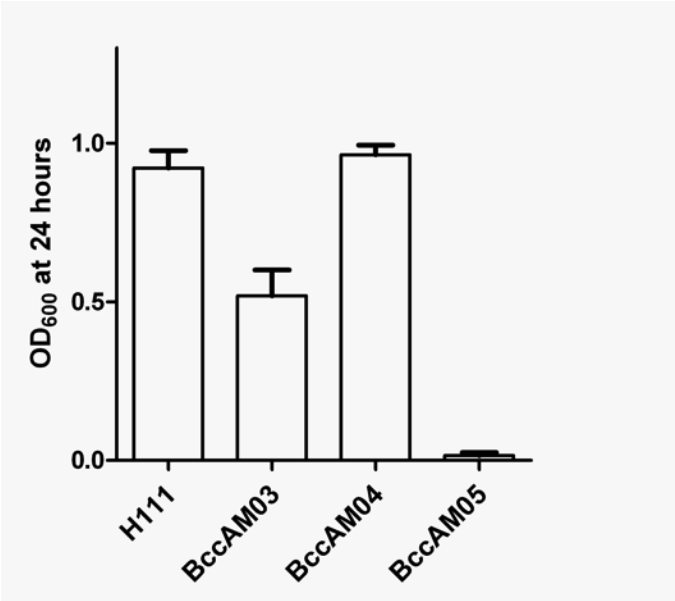


Figure S1: Growth of H111, BccAM03, BccAM04 and BccAM05 in IFS medium, at pH 7 in the presence of 60 µM Bipyridyl, after 24 hours of incubation

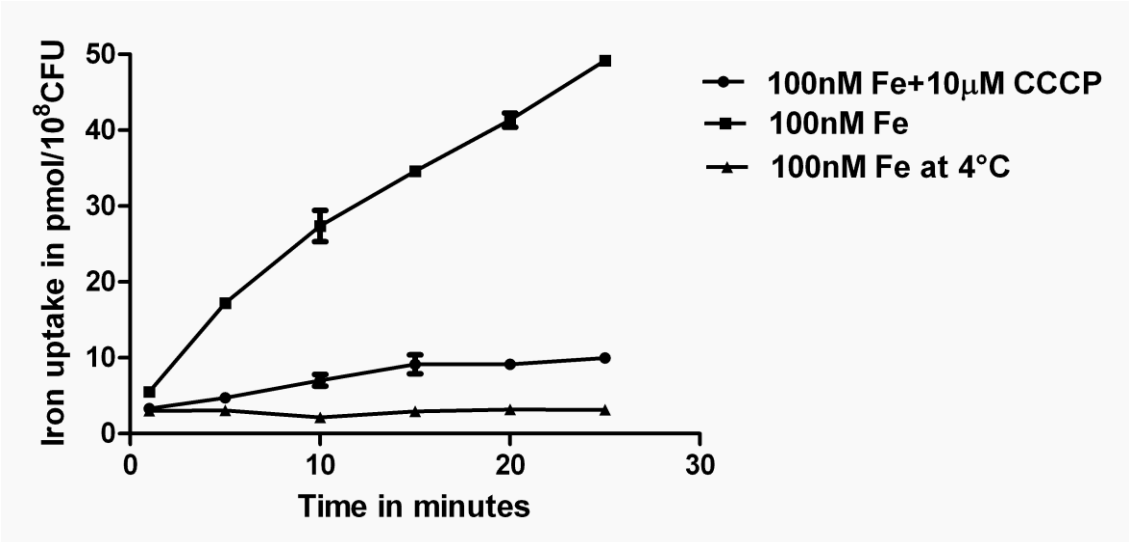


Figure S2: Effect of metabolic inhibitors and low temperature on iron uptake by Ftr_{Bcc}ABCD. Iron uptake of BccAM03 in the presence of CCCP (black circles) and at 4°C (white circles)

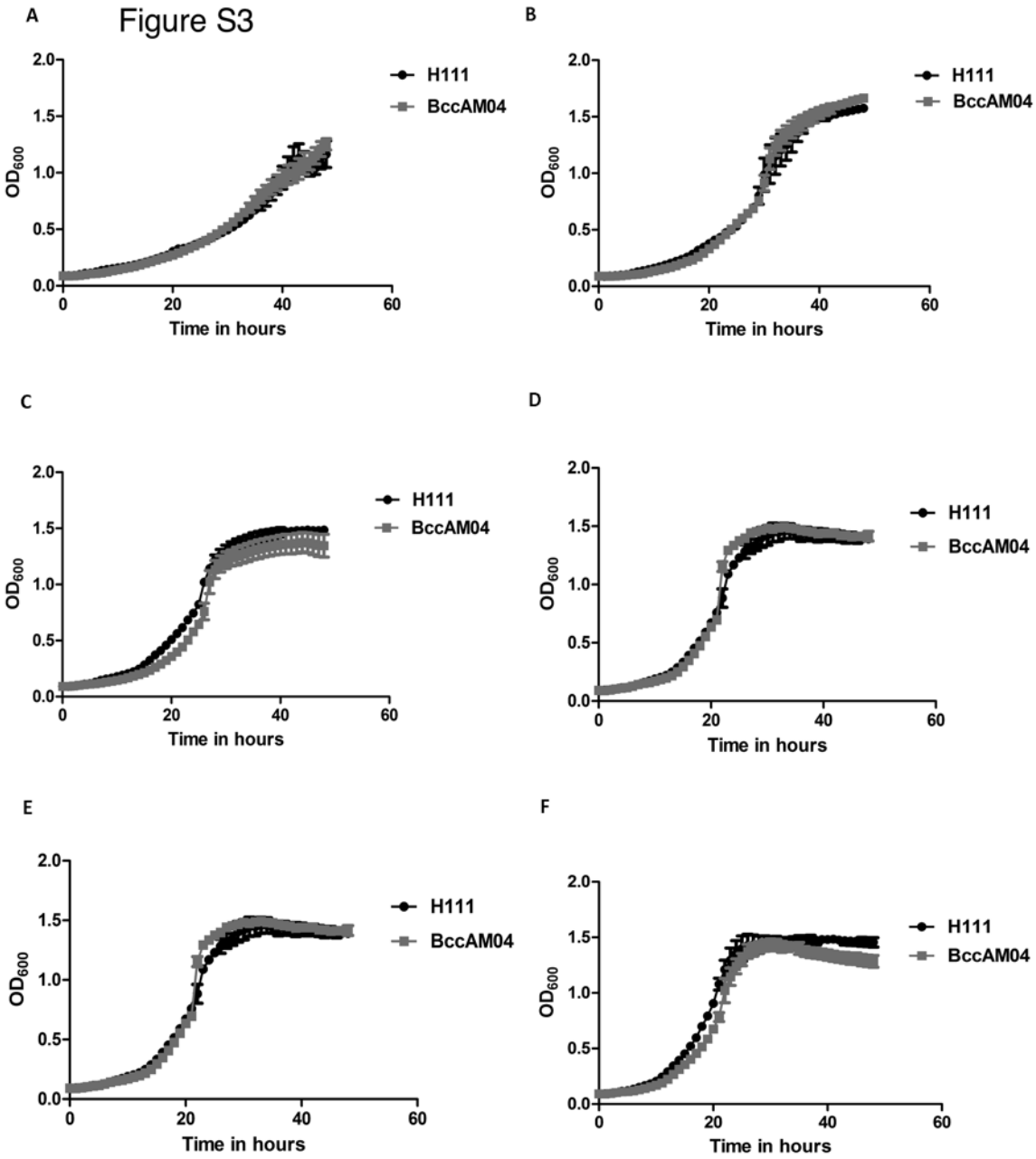


Figure S3: Growth of *B. cenocepacia* H111 and BccAM04 in IFS medium at pH 5 supplemented with ferrous iron: H111 and BccAM04 were grown to late exponential phase and were resuspended in fresh IFS medium at pH 5 supplemented with 1mM ascorbate and with increasing concentrations of ferrous iron: A, 0 nM; B, 100 nM; C, 500 nM; D, 1 µM; E, 5 µM; and F, 10 µM. Data represents the OD_{600} of both strains over a period of 48 hours. IFS medium was supplemented with 2 mM ascorbate and 10 µM desferoxamine in all experimental conditions. Error bars represent the standard error of three independent experiments.

Table S1: Strains and plasmids used in this study

Strains	Genotype/Description	Source/Reference
***B.cenocepacia* strains**		
H111	Clinical isolate, Wild type	(Gotschlich et al., 2001)
BccAM01	H111 Δ*pchAB*	This study
BccAM02	H111 Δ*orbJ*	This study
BccAM03	H111 *ΔpchAB ΔorbJ*	This study
BccAM04	H111 $ftr_{Bcc}C$::pSC200, Tp^R	This study
BccAM05	H111 Δ*pchAB* Δ*orbJ* $ftr_{Bcc}C$ ::pSC200	This study
BccAM06	BccAM03 complemented with pAUM1	This study
BccAM07	BccAM04 complemented with pAUM02	This study
***E.coli* strains**		
Top 10		Invitrogen
Cc118λpir	Δ*(ara, leu)7697 araD*139 Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB(*RfR*) argE(am) recA1 λpir+*	(Herrero et al., 1990)
GR536	K12(W3110) derivative Δ*fec*ABCDE::kan Δ*zup*T::cat Δ*mnt*H Δ*feo*ABC Δ*ent*C	(Grass et al., 2005)
Plasmids		
pBBR1MCS-5	Broad host range vector, Gm^R	(Kovach et al., 1995)
pAUM1	pBBR1MCS-5 with *orb*J	This study
pAUM2	pBBR1MCS-5 with $ftr_{Bcc}ABCD$	This study
pAUM3	pBBR1MCS-5 with $ftr_{Bcc}BCD$	This study
pAUM4	pBBR1MCS-5 with $ftr_{Bcc}ACD$	This study
pAUM5	pBBR1MCS-5 with $ftr_{Bcc}ABD$	This study
pAUM6	pBBR1MCS-5 with $ftr_{Bcc}ABC$	This study

pDONR221	Gateway adapted donor vector; *att*P1 and *att*P2, *ccd*B, pUC origin, Cm^R	Invitrogen
pAUC40	Gateway compatible suicide vector: R6K origin, $Strep^R$ and Cm^R, *sac*B, *ccd*B, *att*R1 and *att*R2	(Carlier et al., 2009)
pSC200	PrhaB rhamnose inducible promoter, *rhaR*, *rhaS* and *dhfr* cassette, Tp^R	(Ortega et al., 2007)
pAUM7	pSC200 containing 300 bp $ftr_{Bcc}C$ fragment	This study

Table S2: Oligonucleotides used in this study

Primer name	Sequence (5' – 3')
Primers used for pyochelin-mutant construction	
*pchAB*_Up_Gw	TACAAAAAAGCAGGCTTCTACCTGCCG ATGATCC
*pchAB*_Up_Kan	GAACTTCGAAGCAGCTCCAGCCTAGGC TTTCACGTACTGCAT
*pchAB*_Dn_Kan	CGGAATAGGAACTAAGGAGGATATTCA TATGGCGCTCGACCATATCCGT
*pchAB*_Dn_Gw	TACAAGAAAGCTGGGTTTTCGATCCGT TGCCGCT
Primers used for ornibactin-mutant construction	
*orbJ*_Up_Gw	TACAAAAAAGCAGGCTCGGATGACGAA GGTGCAA
*orbJ*_Up_Kan	GAACTTCGAAGCAGCTCCAGCCTAACG CATACGCACGATAGAA
*orbJ*_Dn_Kan	CGGAATAGGAACTAAGGAGGATATTCA TATGTCGGCCTGTTCATCAACT
*orbJ*_Dn_Gw	TACAAGAAAGCTGGGTGCCTCCTGTCC CGTTTCC
Primers used for ornibactin mutant complementation *orbJ*_BamH1_comp_fwd *orbJ*_Sma1_comp_rev	 GGGGGATCCGCGATTTCTTCCTGCTCG GGCCCGGGGTAGGTATCGAGCATCGTG
Primers used for *$ftr_{Bcc}C$* conditional mutant	
*$ftr_{Bcc}C$*_fwd	TGGGTCAGATCTTGTTCAT
*$ftr_{Bcc}C$*_rev	CCGTACAGGAAGATCACC
Primers used to amplify *$ftr_{Bcc}ABCD$* locus	
loc_fwd_sma1	GGGCCCGGGATCTTTCCAAGCGTCACT
loc_rev_hind	GGGAAGCTTGAGAGGTGTTGGGCAGTA
Primers used for inverse PCR to construct	

single mutants	
*ftr$_{Bcc}$A*_mut_fwd	GCTCGAGTACGATTTCCC
*ftr$_{Bcc}$A*_mut_rev	GGACACTCCTTGTTATTTG
*ftr$_{Bcc}$B*_mut_fwd	TTTTCGACGATTTCCACC
*ftr$_{Bcc}$B*_mut_rev	CATTCTGGGTTCTCTACG
*ftr$_{Bcc}$C*_mut_fwd	GATCCTCGATGATTCGGG
*ftr$_{Bcc}$C*_mut_rev	CCATCGATACCTCCTGAC
*ftr$_{Bcc}$D*_mut_fwd	GCATGGTGGTTGATGTTC
*ftr$_{Bcc}$D*_mut_rev	GACGATCCACTGGATACC
Primers used to check the components of Ftr$_{Bcc}$ABCD system	
*ftr$_{Bcc}$A*_fwd	TGTTGGGTTCTTCGTTCAT
*ftr$_{Bcc}$A*_rev	TTCGTCAGCTTGTACGTG
*ftr$_{Bcc}$B*_fwd	TGAAAATTCCCCAGAAAATCG
*ftr$_{Bcc}$B*_rev	CTGCACGCTCTCGAATTC
*ftr$_{Bcc}$C*_fwd	TGGGTCAGATCTTGTTCAT
*ftr$_{Bcc}$C*_rev	CCGTACAGGAAGATCACC
*ftr$_{Bcc}$D*_fwd	TGAGCGTTGTCGCCGCCG
*ftr$_{Bcc}$D*_rev	CGCTCACCATCTGTCCGT
Primers used for quantitative PCR	
*ftr$_{Bcc}$A*_qPCR_fwd	CGTACAAGCTGACGAAGCAG
*ftr$_{Bcc}$A*_qPCR_rev Bcal0953 *recA*_fwd Bcal0953 *recA*_rev	ATCGTACTCGAGCGTGATGG GCGATCTTCGACATCCTGTA TTCTCGCCGTTGTAGCTGTA

Table S3: Putative orthologues of the *ftr*$_{Bcc}$*ABCD* genes in *Burkholderiaceae* publicly available genome sequences

Genome	I35_4016	I35_4017	I35_4018	I35_4019
Burkholderia ambifaria AMMD	Bamb_2247	Bamb_2246	Bamb_2245	Bamb_2244
Burkholderia ambifaria IOP40-10	BamIOP4010DRAFT_5318	BamIOP4010DRAFT_5319	BamIOP4010DRAFT_5320	BamIOP4010DRAFT_5321
Burkholderia ambifaria MC40-6	BamMC406_2126	BamMC406_2125	BamMC406_2124	BamMC406_2123
Burkholderia ambifaria MEX-5	BamMEX5DRAFT_5044	BamMEX5DRAFT_5045	BamMEX5DRAFT_5046	BamMEX5DRAFT_5047
Burkholderia cenocepacia AU 1054	Bcen_5868	Bcen_5869	Bcen_5870	Bcen_5871
Burkholderia cenocepacia H111	I35_4016	I35_4017	I35_4018	I35_4019
Burkholderia cenocepacia HI2424	Bcen2424_2209	Bcen2424_2208	Bcen2424_2207	Bcen2424_2206
Burkholderia cenocepacia J2315	BCAL2301	BCAL2300	BCAL2299	BCAL2298
Burkholderia cenocepacia MC0-3	Bcenmc03_2233	Bcenmc03_2232	Bcenmc03_2231	Bcenmc03_2230
Burkholderia cenocepacia PC184	BCPG_02505	BCPG_02506	BCPG_02507	BCPG_02508
Burkholderia dolosa AUO158	BDAG_01103	BDAG_01104	BDAG_01105	BDAG_01106
Burkholderia gladioli BSR3	bgla_1g28870	bgla_1g28860	bgla_1g28850	bgla_1g28840
Burkholderia glumae BGR1	bglu_1g25530	bglu_1g25520	bglu_1g25510	bglu_1g25500
Burkholderia graminis C4D1M	BgramDRAFT_6537	BgramDRAFT_6536	BgramDRAFT_6535	BgramDRAFT_6534
Burkholderia mallei 2002721280	BMA721280_K0008	BMA721280_K0007		BMA721280_K0005
Burkholderia mallei ATCC 10399	BMA10399_G0104	BMA10399_G0105	BMA10399_G0106	BMA10399_G0107
Burkholderia mallei ATCC 23344	BMAA0881	BMAA0882	BMAA0883	BMAA0884
Burkholderia mallei FMH	BMAFMH_E0019	BMAFMH_E0020	BMAFMH_E0021	BMAFMH_E0022
Burkholderia mallei GB8 horse 4	BMAGB8_A0982	BMAGB8_A0983	BMAGB8_A0984	BMAGB8_A0985
Burkholderia mallei JHU	BMAJHU_I0017	BMAJHU_I0018	BMAJHU_I0019	BMAJHU_I0020
Burkholderia mallei NCTC 10229	BMA10229_0014	BMA10229_0013	BMA10229_0012	BMA10229_0011
Burkholderia mallei NCTC 10247	BMA10247_A0914	BMA10247_A0913	BMA10247_A0912	BMA10247_A0911
Burkholderia mallei PRL-20	BMAPRL20_0933	BMAPRL20_0932	BMAPRL20_0931	BMAPRL20_0930
Burkholderia mallei SAVP1	BMASAVP1_1740	BMASAVP1_1741	BMASAVP1_1742	BMASAVP1_1743
Burkholderia multivorans ATCC 17616	Bmul_1068	Bmul_1069	Bmul_1070	Bmul_1071

Table S3: Putative orthologues of the *ftr$_{Bcc}$ABCD* genes in *Burkholderiaceae* publicly available genome sequences (continued)

Burkholderia multivorans CGD1	BURMUCGD1_1011	BURMUCGD1_1012	BURMUCGD1_1013	BURMUCGD1_1014
Burkholderia multivorans CGD2	BURMUCGD2_1398	BURMUCGD2_1399	BURMUCGD2_1400	BURMUCGD2_1401
Burkholderia multivorans CGD2M	BURMUCGD2M_1495	BURMUCGD2M_1496	BURMUCGD2M_1497	BURMUCGD2M_1498
Burkholderia oklahomensis C6786	BoklC_010100020444	BoklC_010100020449	BoklC_010100020454	BoklC_010100020459
Burkholderia oklahomensis EO147	BoklE_010100021022	BoklE_010100021027	BoklE_010100021032	BoklE_010100021037
Burkholderia phymatum STM815	Bphy_1957	Bphy_1956	Bphy_1955	Bphy_1954
Burkholderia phytofirmans PsJN	Bphyt_1397	Bphyt_1398	Bphyt_1399	Bphyt_1400
Burkholderia pseudomallei 1106a	BURPS1106A_A0508	BURPS1106A_A0509	BURPS1106A_A0510	BURPS1106A_A0511
Burkholderia pseudomallei 1106b	BURPS1106B_1490	BURPS1106B_1489	BURPS1106B_1488	BURPS1106B_1487
Burkholderia pseudomallei 1655	BURPS1655_0372	BURPS1655_0373	BURPS1655_0375	BURPS1655_0379
Burkholderia pseudomallei 1710a	BURPS1710A_A3002	BURPS1710A_A3003	BURPS1710A_A3004	BURPS1710A_A3005
Burkholderia pseudomallei 1710b	BURPS1710b_A1921	BURPS1710b_A1922	BURPS1710b_A1923	BURPS1710b_A1924
Burkholderia pseudomallei 305	BURPS305_7937	BURPS305_7936	BURPS305_7935	BURPS305_7934
Burkholderia pseudomallei 406e	BURPS406E_O0199	BURPS406E_O0198	BURPS406E_O0197	BURPS406E_O0196
Burkholderia pseudomallei 576	BUC_4662	BUC_4663	BUC_4664	BUC_4665
Burkholderia pseudomallei 668	BURPS668_A0606	BURPS668_A0607	BURPS668_A0608	BURPS668_A0609
Burkholderia pseudomallei K96243	BPSS0357	BPSS0358	BPSS0359	BPSS0360
Burkholderia pseudomallei MSHR346	GBP346_B3181	GBP346_B3182	GBP346_B3183	GBP346_B3184
Burkholderia pseudomallei Pakistan 9	BUH_4800	BUH_4801	BUH_4802	BUH_4803
Burkholderia pseudomallei Pasteur 52237	BURPSPAST_D0102	BURPSPAST_D0141	BURPSPAST_D0163	BURPSPAST_D0172
Burkholderia pseudomallei S13	BURPSS13_L0010	BURPSS13_L0011	BURPSS13_L0012	BURPSS13_L0013
Burkholderia rhizoxinica HKI 454	RBRH_02876	RBRH_02877	RBRH_02878	RBRH_02879
Burkholderia sp. 383	Bcep18194_A5537	Bcep18194_A5536	Bcep18194_A5535	Bcep18194_A5534
Burkholderia sp. CCGE1001	BC1001_1139	BC1001_1140	BC1001_1141	BC1001_1142

Table S3: Putative orthologues of the *ftr$_{Bcc}$ABCD* genes in *Burkholderiaceae* publicly available genome sequences (continued)

Burkholderia sp. CCGE1002	BC1002_1014	BC1002_1015	BC1002_1016	BC1002_1017
Burkholderia sp. CCGE1003	BC1003_2269	BC1003_2268	BC1003_2267	BC1003_2266
Burkholderia sp. Ch1-1	BCh11DRAFT_6517	BCh11DRAFT_6518	BCh11DRAFT_6519	BCh11DRAFT_6520
Burkholderia sp. H160	BH160DRAFT_5673	BH160DRAFT_5672	BH160DRAFT_5671	BH160DRAFT_5670
Burkholderia thailandensis Bt4	BthaB_010100011510	BthaB_010100011505	BthaB_010100011500	BthaB_010100011495
Burkholderia thailandensis E264	BTH_II2035	BTH_II2034	BTH_II2033	BTH_II2032
Burkholderia thailandensis E264	BthaA_010200016863	BthaA_010200016858	BthaA_010200016853	BthaA_010200016848
Burkholderia thailandensis TXDOH	BthaT_010100011888	BthaT_010100011883	BthaT_010100011878	BthaT_010100011873
Burkholderia vietnamiensis G4	Bcep1808_2294	Bcep1808_2293	Bcep1808_2292	Bcep1808_2291
Burkholderia xenovorans LB400	Bxe_A3152	Bxe_A3151	Bxe_A3150	Bxe_A3149
Candidatus Burkholderia kirkii UZHbot1	BKIR_c170_1734	BKIR_c170_1735	BKIR_c170_1736	BKIR_c170_1737
Achromobacter arsenitoxydans SY8	KYC_10593	KYC_10588	KYC_10583	KYC_10578
Achromobacter piechaudii ATCC 43553	HMPREF0004_2125	HMPREF0004_2126	HMPREF0004_2127	HMPREF0004_2128
Achromobacter xylosoxidans A8	AXYL_01669	AXYL_01670	AXYL_01671	AXYL_01672
Achromobacter xylosoxidans AXX-A	AXXA_17941	AXXA_17946	AXXA_17951	AXXA_17956
Acidovorax avenae subsp. avenae ATCC 19860				
Acidovorax citrulli AAC00-1				
Acidovorax delafieldii 2AN				
Acidovorax sp. JS42				
Acidovorax sp. NO-1				
Alicycliphilus denitrificans BC				
Alicycliphilus denitrificans K601				
Aromatoleum aromaticum EbN1	ebA1861			ebA1869
Bordetella avium 197N	BAV1224	BAV1225	BAV1226	BAV1227

Table S3: Putative orthologues of the *ftr*$_{Bcc}$*ABCD* genes in *Burkholderiaceae* publicly available genome sequences (continued)

Bordetella bronchiseptica RB50	BB3593	BB3592	BB3591	3590
Bordetella parapertussis 12822	BPP3193	BPP3192	BPP3191	BPP3190
Bordetella pertussis CS	BPTD_1144	BPTD_1145	BPTD_1146	BPTD_1147
Bordetella pertussis Tohama I	BP1152	BP1153	BP1154	BP1155
Bordetella petrii DSM 12804	Bpet1852	Bpet1853	Bpet1854	Bpet1855
Burkholderiales bacterium 1_1_47				
Candidatus Accumulibacter phosphatis clade IIA str. UW-1				
Candidatus Zinderia insecticola CARI				
Collimonas fungivorans Ter331	CFU_1796	CFU_1797	CFU_1798	CFU_1799
Comamonas testosteroni ATCC 11996				
Comamonas testosteroni CNB-2				
Comamonas testosteroni KF-1				
Comamonas testosteroni S44				
Cupriavidus basilensis OR16				
Cupriavidus necator N-1				
Cupriavidus taiwanensis				
Delftia acidovorans SPH-1				
Delftia sp. Cs1-4				
Diaphorobacter sp. TPSY				
Herbaspirillum seropedicae SmR1	Hsero_2722	Hsero_2721	Hsero_2720	Hsero_2719
Herminiimonas arsenicoxydans				
Hylemonella gracilis ATCC 19624				
Janthinobacterium sp. Marseille				
Lautropia mirabilis ATCC 51599				
Leptothrix cholodnii SP-6				

Table S3: Putative orthologues of the *ftr*$_{Bcc}$*ABCD* genes in *Burkholderiaceae* publicly available genome sequences (continued)

Limnobacter sp. MED105
Oxalobacter formigenes HOxBLS
Oxalobacter formigenes OXCC13
Oxalobacteraceae bacterium IMCC9480
Parasutterella excrementihominis YIT
11859
Polaromonas naphthalenivorans CJ2
Polaromonas sp. JS666
Polynucleobacter necessarius subsp.
asymbioticus QLW-P1DMWA-1
Polynucleobacter necessarius subsp. necessarius STIR1
Pusillimonas sp. T7-7
Ralstonia eutropha H16
Ralstonia eutropha JMP134
Ralstonia metallidurans CH34
Ralstonia pickettii 12D
Ralstonia pickettii 12J
Ralstonia solanacearum CFBP2957
Ralstonia solanacearum GMI1000
Ralstonia solanacearum Po82
Ralstonia solanacearum PSI07
Ralstonia solanacearum UW551
Ralstonia sp. 5_2_56FAA
Ralstonia sp. 5_7_47FAA
Ramlibacter tataouinensis TTB310
Rhodoferax ferrireducens T118

Table S3: Putative orthologues of the *ftr*$_{Bcc}$*ABCD* genes in *Burkholderiaceae* publicly available genome sequences (continued)

Rubrivivax benzoatilyticus JA2	RBXJA2T_00010	RBXJA2T_00020		RBXJA2T_00030
Rubrivivax gelatinosus IL144	RGE_22270	RGE_22250		RGE_22230
Sutterella parvirubra YIT 11816				
Sutterella wadsworthensis 3_1_45B				
Taylorella asinigenitalis MCE3				
Taylorella equigenitalis MCE9				
Thiomonas intermedia K12				
Variovorax paradoxus EPS				
Variovorax paradoxus S110				
Verminephrobacter eiseniae EF01-2	Veis_3603	Veis_3604	Veis_3605	Veis_3606

Table S4: Siderophore production by *Burkholderia* isolates

Strain name	Strain ID	Siderophore production after 72 hours
Burkholderia silvatlantica	LMG23149	-/+
Burkholderia ferrariae	LMG23612	+
Burkholderia mimosarum	LMG23256	-
Burkholderia sacchari	LMG19450	-
Burkholderia nodosa	LMG23741	+
Burkholderia unamae	LMG22722	+
Burkholderia tropica	LMG22274	+
Burkholderia bannensis	LMG26378	+
Burkholderia kururiensis	LMG19447	+
Burkholderia tuberum	LMG21444	-
Burkholderia semidicola	LMG24236	+
Burkholderia phenazinium	LMG2247	+
Burkholderia sartisoli	LMG24000	-
Burkholderia hospita	LMG20598	-/+
Burkholderia caribensis	LMG18531	+
Burkholderia phymatum	LMG21445	+
Burkholderia phytofirmans	LMG22487	+
Burkholderia megapolitana	LMG23650	-/+
Burkholderia ginsengisoli	LMG24044	-/+
Burkholderia bryophila	1S18	+
Burkholderia xanthomonas	LMG21463	+
Burkholderia terricola	LMG20594	+
Burkholderia caledonica	LMG19079	+
Burkholderia fungorum	LMG16225	+
Burkholderia phenoliruptrix	LMG22037	+
Burkholderia graminis	LMG18924	+
Burkholderia glumae	LMG2196	+
Burkholderia plantarii	LMG9036	+
Burkholderia gladioli	LMG2216	+
Burkholderia andropogonis	LMG2129	-
Burkholderia claryophylli	LMG2155	+
Burkholderia sordidicola	LMG22029	+
Burkholderia glathei	LMG14190	+

Siderophore production was tested by spotting bacterial suspensions and CAS medium and incubating at 30°C for 72h (See Material and Methods for details). Legend: +, easily detectable after 72 hours of incubation; ±, detectable at very low amounts after 72 hours; -, not detectable

3.6 Unpublished results

While I was in the process of investigating the Ftr system in *B. cenocepacia* H111, similar systems were described in *Bordetella pertussis* and *Brucella abortus*. Although we conducted different sets of experiments to those described in these publications, redundant data have been removed from our manuscript. Such findings and additional experiments are described below.

Bioinformatics- Ftr system

The ability of the siderophore mutants of H111 to grow in iron-limited conditions suggested the presence of an alternative iron uptake system. This prompted us to search the H111 genome annotation for putative iron transport systems and we encountered a potential locus comprised of 4 open reading frames, which we named $ftr_{Bcc}ABCD$ (Locus tag: I35_4016 – I35_4019); (Mathew *et al*., 2014). The four components of the locus, encoded domains and their homologs are listed in Table 4. The proteins encoded by the $ftr_{Bcc}ABCD$ operon share more than 50 % sequence identity with the recently described FtrABCD system of *Bordetella pertussis* (Brickman and Armstrong, 2012).

The predicted FtrC protein consists of 268 amino acids with a molecular weight of 30.68 kDa. It is annotated as a putative iron permease belonging to the FTR1 family of integral membrane iron transporters (Kosman, 2003). The best characterised member of this protein family is FTR1 of *Saccharomyces cerevisiae*, which interacts with a multicopper oxidase and mediates high-affinity iron uptake (Stearman *et al*., 1996). Sequence alignment shows that FtrC shares 26 % and 25 % identity to the characterized iron uptake proteins FTR1 of *Saccharomyces cerevisiae* and EfeO of *Escherichia coli*, respectively. The role of these FTR1-family proteins in iron uptake has been experimentally confirmed in *S. cerevisiae* (Stearman *et al*., 1996) and *E. coli* (Grosse *et al*., 2006; Cao *et al*., 2007). The TMHMM program predicted that FtrC has

7 transmembrane domains. Two REXXE motifs shown to be essential for iron binding (Stearman *et al*., 1996; Severance *et al*., 2004) are located on the first and fourth transmembrane domains of FtrC as in the yeast and other bacterial homologs.

The *ftrB* gene, located immediately upstream of *ftrC*, encodes a putative periplasmic cupredoxin. The occurrence of a cupredoxin domain adjacent to an FTR1-family protein follows a similar arrangement in yeast, where a multicopper oxidase is also part of the FTR1 iron uptake system. Moreover, FtrB shares 21 % similarity with EfeO, the cupredoxin component of the EfeUOB system of *E.coli*. A previous analysis of different EfeO-Cup domain proteins revealed that approximately 100 EfeO homologs in the bacterial sequence databases, including FtrB, are associated with iron transport-related genes (Rajasekaran *et al*., 2010). However FtrB differs from the EfeO protein in that it lacks the C-terminal peptidase-M75 domain (Rajasekaran *et al*., 2010).

Adjacent to *ftrB* in H111 is *ftrA*, which encodes a putative 19kDa periplasmic protein. FtrA does not show sequence similarity with any components of the EfeUOB system of *E. coli* or the FTR1/FET3 system of *S. cerevisiae*. Instead, the predicted FtrA protein shares 53 % sequence identity with protein P19 of *Campylobacter jejuni* (Chan et al., 2010) and 51 % identity with the FetP protein of the FetMP system, another iron uptake system recently identified in *E. coli* (Koch *et al*., 2011). P19 from *C. jejuni* has been shown to be required for growth under iron limiting conditions (Chan et al., 2010). FetP of *E. coli* enhanced iron uptake at low ferrous iron concentrations and at higher ferric iron concentrations. This has been attributed to its dual role as a periplasmic ferrous iron binding protein and a ferric reductase (Koch *et al*., 2011). A P19 homologue is also found in the magnetotactic marine *Vibrio* sp. MV-1, and is perhaps involved in iron uptake and therefore in the formation of magnetosomes (Dubbels *et al*., 2004).

The fourth component of the $ftr_{Bcc}ABCD$ operon encodes the FtrD protein, which is a putative ferredoxin with 9 transmembrane domains. Ferredoxins are iron sulphur proteins generally involved in electron transfer reactions. The predicted FtrD protein exhibits 28 % sequence identity with the ferredoxin of *Vibrio* sp.MV-1, which is adjacent to the gene encoding the P19 protein implicated in iron uptake (Dubbels *et al*., 2004).

ORFs in the locus	Putative function	Homologs
ftrA	**Periplasmic protein involved in high affinity ferrous iron transport**	**P19 in *C.jejuni*** ***fetP* in *E.coli*** ***ftrA* in *B.pertussis***
ftrB	**Cupredoxin**	***efeO* in *E.coli*** ***ftrB* in *B.pertussis***
ftrC	**High affinity iron permease**	***ftr1* in *S.cerevisiae*** ***efe*U in *E.coli*** ***ftrC* in *B.pertussis***
ftrD	**Ferredoxin**	***ftrD* in *B.pertussis***

Table 4: Putative functions and homolgs of ORFs in ftrABCD locus

Ftr_{Bcc}ABCD is a specific transport system for iron

We demonstrated that the Ftr_{Bcc}ABCD system transports iron, but the possibility remains that iron may not be the primary substrate of the transporter. We therefore tested if other divalent metals such as manganese, copper, magnesium and zinc affect iron transport via the Ftr_{Bcc}ABCD system in strain BccAM03. We found that none of the divalent metals tested could out-compete iron for transport (Figure 12). Only 10 µM $CuSO_4$ showed an effect on iron uptake, but the label content of cells decreased over time, indicating a cytotoxic effect of copper. Addition of manganese and zinc increased iron uptake in BccAM03, possibly by inducing the expression of the *ftrABCD* genes. We conclude from these results that Ftr_{Bcc}ABCD is a specific iron transporter rather than an nonspecific metal transporter.

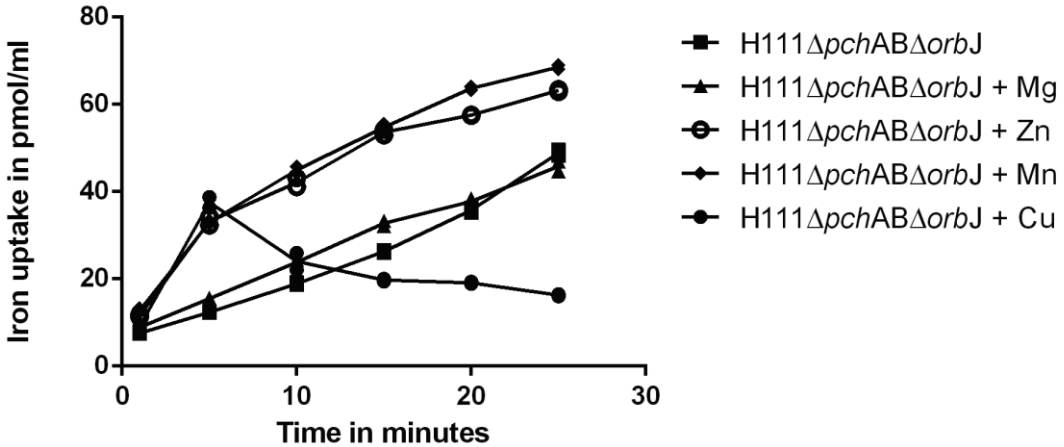


Figure 12: Effect of various divalent cations on transport of iron by the Ftr_{Bcc}ABCD system. Uptake assays were initiated by adding 1 µM $^{59}FeCl_3$ and 10 µM of corresponding metals to the cultures. At 5-min intervals, 1-ml samples of the culture were removed and $^{59}FeCl_3$ taken up by the cells were

quantified using a Packard liquid scintillation counter. Representative data from one of two independent experiments are shown.

$Ftr_{Bcc}ABCD$ is a high affinity iron transporter

Although we examined the kinetics of the Ftr system in a heterologous *E. coli* host, and this is described in our manuscript, the initial analysis was carried out using H111 and different mutants. We measured $FeCl_3$ uptake kinetics of H111, BccAM04, BccAM03 and BccAM05 grown in iron limited succinate medium (Figure 13). The kinetic parameter, Michaelis-Menten constant value (Km) is shown in Table 5. The H111 wild-type strain exhibited the lowest Km value of 0.7 µM. The mutant BccAM03, lacking both siderophores but possessing a functional $Ftr_{Bcc}ABCD$ system, also displayed a low Km Value of 1.1 µm, indicating a high affinity of the $Ftr_{Bcc}ABCD$ system for $FeCl_3$. The BccAM04 strain, lacking a functional $Ftr_{Bcc}ABCD$ system, had a Km value of 1 µM. This Km value is almost equal to that of the BccAM03 strain and suggests that the $Ftr_{Bcc}ABCD$ has a similar affinity for iron as the siderophore systems. Finally, BccAM05, lacking all putative $FeCl_3$ uptake systems, had a high Km value of 8 µm. The fact that this Km value is not higher suggests the presence of a low affinity iron uptake system in H111.

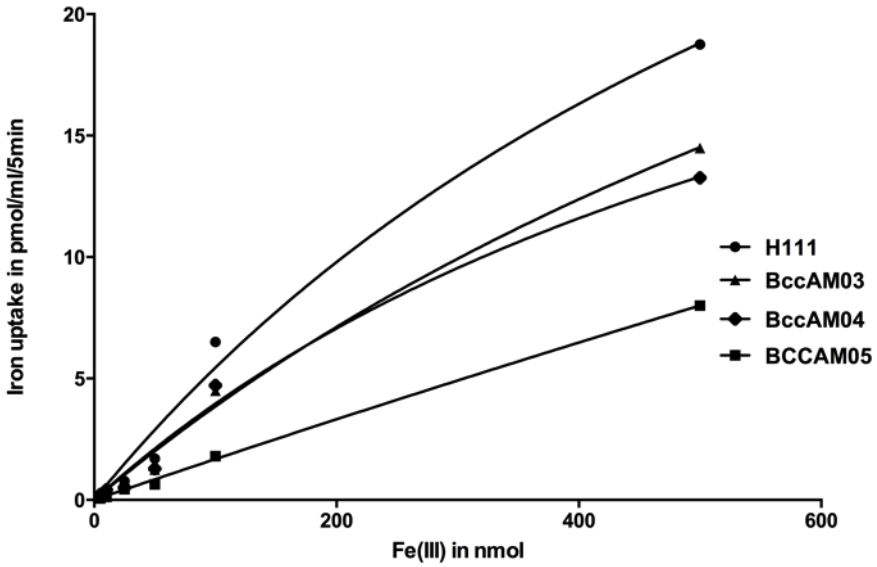


Figure 13: Kinetics of iron uptake by the $Ftr_{Bcc}ABCD$ system (A) Hyperbolic plot of kinetic data for the transport of $^{59}FeCl_3$ by H111 (black circles), BccAM03 (black triangles), BccAM04 (white diamonds) and BccAM05 (black squares). Strains grown at 37 °C in iron limited succinate medium were assayed for uptake in various concentrations of $^{59}FeCl_3$ ranging from 10 nM to 500 nM. The initial iron uptake rate was determined from the iron uptake slope and has been plotted against increasing iron concentrations. The data was fitted to the Michaelis-Menten equation using Prism software from Graphpad and Km values calculated using the same software.

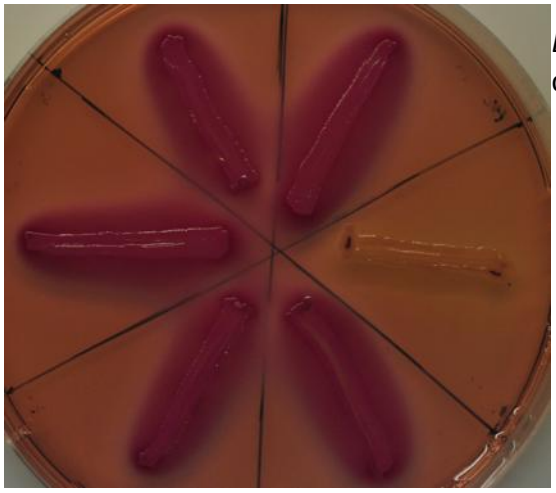
Strain	Km (µm)
H111	0.7
BccAM03	1.1
BccAM04	1
BccAM05	8

Table 5: Kinetic parameter of iron uptake by H111 and mutants

Ftr_{Bcc}ABCD is under the control of the iron regulator Fur

We demonstrated that the Ftr_{Bcc}ABCD system is regulated by iron concentration in the medium. However whether this regulation is mediated by the Fur repressor protein as in other iron transport systems was not known. To test this, a fur titration assay was performed as described previously (Stojiljkovic et al., 1994). This assay was done using the *E. coli* strain H1717, which carries a Fur-regulated chromosomal *fhuF-lacZ* gene fusion, which is sensitive to changes in the concentration of the Fur repressor. Under iron replete conditions, Fur represses transcription of *lacZ*, leading to a *lacZ*$^-$ phenotype. Fur boxes introduced on a multicopy plasmid can relieve fur repression by titrating the Fur protein away from the single chromosomal Fur box, thus allowing increased utilization of lactose.

To perform the assay, approximately 1.3kb *ftr* promoter region upstream of *ftrA* was cloned into the high copy number plasmid, pBluescriptII KS and introduced by transformation into *E. coli* top 10, which was then transferred into *E. coli* H1717. The clones were plated onto the differential medium, MacConkey agar containing 40 µM $Fe(NH_4)_2(SO_4)_2$ which resulted in pink colonies (Figure 14). This finding indicated that the plasmid with the *ftr* promoter region carries a Fur-binding fragment, which relieves *lacZ* repression, therefore resulting in a lac$^+$ phenotype. H1717 with the plasmid p3ZFBS containing the *E. coli* consensus Fur box and H1717 containing the empty plasmid pBluescriptII KS were used as positive and negative controls respectively. *E. coli* H1717 and the positive control plasmid were kindly provided by Dr. Mark S Thomas, Sheffield, England.



E. coli H1717/p3ZFBS(Positive control)

E. coli H1717::pBluescript (Negative control)

Figure 14: Fur titration assay: plate showing lac- phenotype of the negative control *E. coli* H1717::pBluescriptII KS, lac+ phenotype of positive control, *E. coli* H1717::p3ZFBS and lac+ phenotypes of 4 different clones of H1717 with ftr promoter region

Gluconate may act as a ligand to transport iron through Ftr system

Since we observed that the Ftr system could transport iron in its ferric form, we hypothesized that certain ligands are involved in binding and transporting ferric iron into the cell. It has been shown that *Burkholderia* species are able to produce organic acids such as gluconate, which could be an effective ligand of iron (Song *et al*., 2008). To test whether gluconate is involved in solubilisation and transport of ferric iron, the ability of *E. coli* GR536 harboring the Ftr system (*E. coli* GR536 (pAUM2)) to acquire iron when hematite was provided as the sole iron source was analysed in the presence of gluconate as well as culture supernatant of *B. cenocepacia* H111. Hematite appeared to bind free iron in the M9 medium thus inhibiting growth of *E. coli* strain in this experiment. However, gluconate appeared to induce iron transport from hematite in *E. coli* GR536 (pAUM2) thereby complementing the growth defect. Likewise, filtered supernatant of H111 siderophore mutant imparted a similar growth effect on *E. coli* GR536, indicating that H111 secretes ligands other than siderophores, which can bind and transport

iron through the Ftr system (Figure 15). However the iron ligand in the H111 siderophore mutant culture supernatant is yet to be identified.

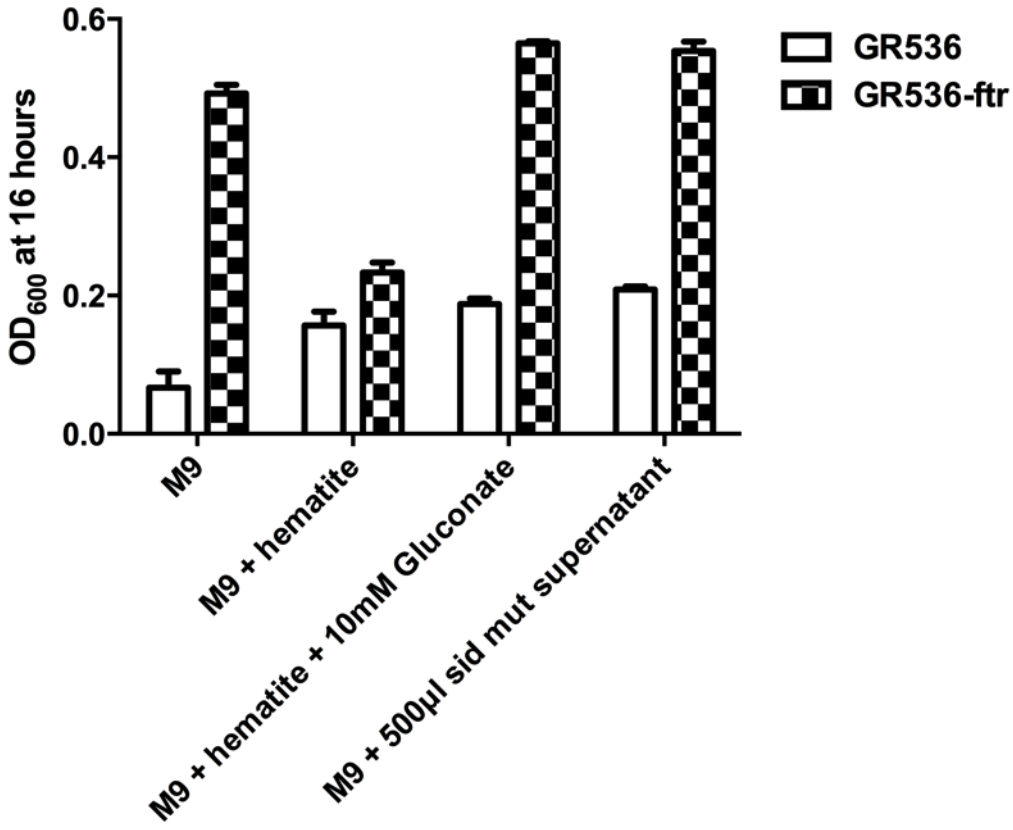


Figure 15: Growth of *E. coli* GR536 and *E. coli* GR536 (pAUM2) in M9 medium. 0.01 mg hematite was added to the cultures. The cultures were additionally supplemented with 10 mM gluconate or 500 µl of supernatant from H111 siderophore mutant cultures.

ftrABCD locus encodes a reductase

We observed previously that the Ftr system could transport ferric iron, which was then subsequently reduced to ferrous iron. To investigate whether *ftr* locus encodes a ferric reductase and to identify the reductase component, a ferric reductase assay was performed using the *E. coli* heterologous system as previously described with some modifications (Georgatsou & Alexandraki, 1994). Briefly, GR536 (pAUM2), GR536 (pAUM3), GR536 (pAUM4), GR536 (pAUM5) and GR536 (pAUM6) cells were grown to an OD600 of approximately 0.5, subsequently washed twice in IFS medium

and resuspended to a concentration of approximately 5 X 10^6 cells per ml in the assay buffer containing 50 mM sodium citrate (pH 4) and 0.5 % glucose. The cells were then incubated with 1 mM ferrous iron chelator, bathophenanthroline disulfonite (BPDS) and different concentrations of ferric iron ranging from 1-100 µM of ferric chloride for 1 hour and absorbance at 520nm was recorded. The amount of ferrous ions produced was estimated by a calibration curve using a solution of known ferrous ion concentrations. Ferric reductase activity was expressed in nmol of ferrous ions produced under the assay conditions. *E. coli* GR536 strain was involved in the assay to rule out the background reductase activity

We observed that *E. coli* GR536 (pAUM2) harboring the Ftr system exhibited reductase activity under assay conditions. However, no such reductase reaction was observed with *E. coli* GR536 strains lacking single components of the Ftr system (Figure 16). This could be due to the fact that the Ftr system works as a complex and inactivation of one component leads to the inhibition of activity of the entire complex.

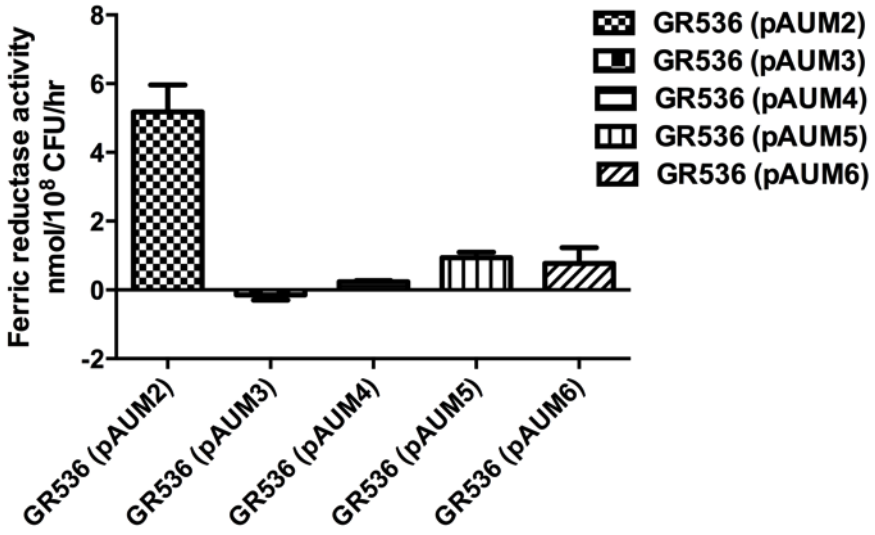


Figure 16: Ferric reductase assay employing *E. coli* GR536 harboring Ftr system. 1 µM ferric iron and 1 mM ferrous iron chelator BPDS was added to the cultures and incubated for 1 hour at 37°C. Bars indicate amount of ferrous iron produced which indicates the ferric reductase activity of the Ftr system. Ferrous iron generated by GR536 strain was subtracted to remove the background reductase activity

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4. Results II

Chapter 4. Results II: Ornibactin production increases the pathogenic potential of the environmental isolate, *Burkholderia sacchari*

4.1 Introduction

Burkholderia are a diverse group of Gram-negative β-proteobacteria that range from plant beneficial strains to notorious human pathogens (Coenye & Vandamme, 2003; Mahenthiralingam *et al*., 2005). It has recently been suggested that the genus *Burkholderia* can be divided into two clades: one containing the pathogenic *Burkholderia* species such as *B. mallei* and *B. ps*eudomallei as well as the 17 species of the *Burkholderia cepacia* complex (BCC), and the other containing non-pathogenic *Burkholderia* species that are mostly associated with plants and are considered to be beneficial (Suárez-Moreno *et al*., 2012). The *pseudomallei* group includes primary pathogens of humans and animals while the 17 BCC species comprise opportunistic pathogens that have been isolated from diverse niches (Mahenthiralingam *et al*., 2000; Vermis *et al*., 2002; Vanlaere *et al*., 2008; Mahenthiralingam *et al*., 2006). The vast majority of remaining *Burkholderia* species are mainly associated with plants or soil. Many of them have been recognised for their potential biotechnological applications, such as plant growth promotion, phytoremediation, and biocontrol (Hebbar *et al*., 1998; Tran Van *et al*., 2000). Strains of the plant beneficial group of *Burkholderia* could be valuable in field applications, particularly as these strains have never been associated with human or plant infections.

However, the fact that pathogenic and non-pathogenic *Burkholderia* strains have been continuously isolated from the rhizosphere suggests that soil can be a reservoir for many potential pathogens. Transfer of pathogenic islands in such a context has been described in many bacterial species (Dobrindt *et al*., 2004; Aminov, 2011) and such a possibility cannot be ruled out. It has been suggested that factors important for the survival of many environmental strains could generate highly adaptable strains that can also infect humans (Paganin *et al*., 2011). It has also been postulated that traits required for adaptation to different environmental conditions contribute more significantly to chronic rather than acute infections (Loutet & Valvano, 2010).

This suggests that a harmless strain can also reveal its pathogenic potential when exposed to different niches or when chromosomal changes occur due to gene or pathogenicity determinant acquisition. This scenario is particularly important in *Burkholderia* species, as several *Burkholderia* strains have evolved to become opportunistic pathogens over the years (Eberl & Tümmler, 2004; Mahenthiralingam *et al*., 2008; Mahenthiralingam *et al*., 2005). However, a recent analysis of genomes of several symbiotic and environmental *Burkholderia* strains demonstrated that they do not possess most of the virulence determinants and are therefore unlikely to pose a threat to humans (Angus *et al*., 2014).

Efficient assimilation of essential nutrients is one of the main drivers of persistence in the environment. Among them, iron is an essential trace element and consequently organisms have evolved various strategies for efficient uptake of this metal. Production of siderophores is the most important mechanism employed by pathogens for efficient transport of iron (Guerinot, 1994; Neilands, 1995). *Burkholderia* also produces several siderophores and, among them, ornibactin has been shown to be particularly important for virulence of Bcc strains in different infection models (Thomas, 2007; Visser *et al*., 2004; Uehlinger *et al*., 2009). However, the importance of siderophores for growth of *Burkholderia* species in other habitats has not been investigated.

Siderophores have been proposed to be beneficial for soil bacteria, considering the competitive advantage of producing strains over non-producers in iron-limited habitats, as well as their ability to suppress the growth of other phytopathogens (Caballero-Mellado *et al*., 2007; Lugtenberg & Kamilova, 2009). We have been studying iron transport mechanisms of *Burkholderia* and observed that some prominent environmental *Burkholderia* isolates did not produce siderophores when tested on CAS plates (Mathew *et al*., 2014). This finding called into question the importance of siderophores for iron acquisition in these plant and rhizosphere associated *Burkholderia* strains and we therefore investigated whether siderophores play an important role in the ecophysiology of *Burkholderia*

To this end, we introduced the ornibactin biosynthesis genes into the legume nodulating species *B. tuberum*, and the rhizosphere isolate *B. sacchari* and analysed their fitness in various habitats. *B. tuberum* did not show any significant increase in pathogenic potential, even when producing siderophores. However, ornibactin production gave *B. sacchari* the ability to colonise the infection model *G. mellonella* and induce symptoms typical of Bcc infection.

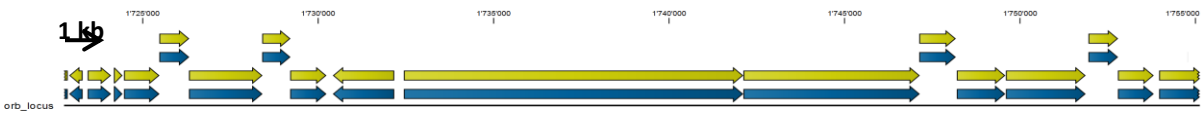
4.2 Results

4.2.1 Construction of transgenic *B. tuberum* and *B. sacchari* strains that produce ornibactin

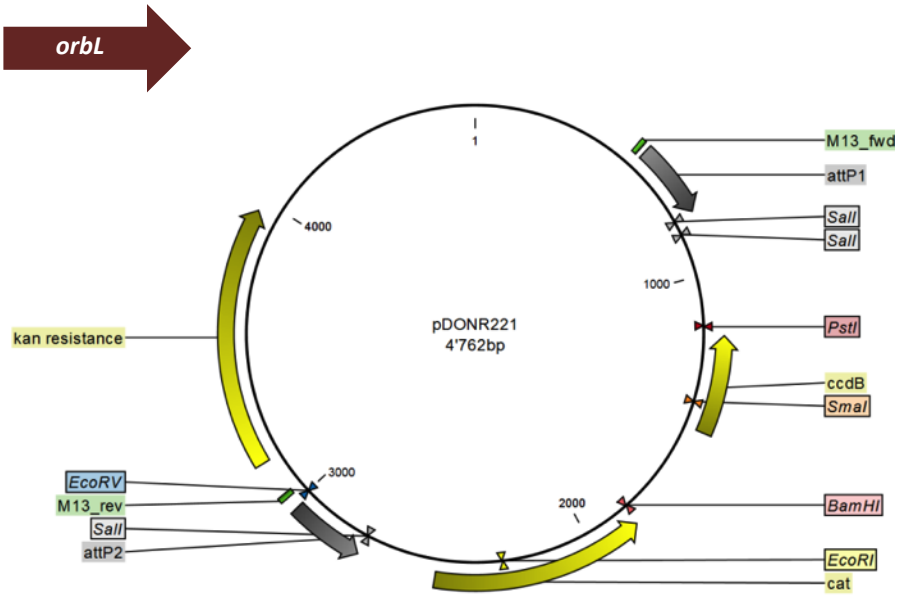
The ornibactin locus from *B. cenocepacia* H111 was cloned and inserted in to *B. tuberum* and *B. sacchari* strains (Figure 17) using the modified oriTn7 capture system developed for *B.pseudomallei* (Kvitko, *et al.*, 2013). The introduction of the ornibactin biosynthesis and transport gene cluster at the established attTn7 site was confirmed by PCR and sequencing using primers for the adjacent *glmS* gene and the Tn7 region. The recombinant strains showed siderophore activity when tested on CAS plates as shown in Figure 18 & 19 and ornibactin production was confirmed by extracting the siderophore from the culture supernatant of these strains. Introduction of ornibactin induced the growth of *B. tuberum* and *B. sacchari* strains in iron limited conditions in the presence of the iron chelator, desferoxamine (Figure 20)

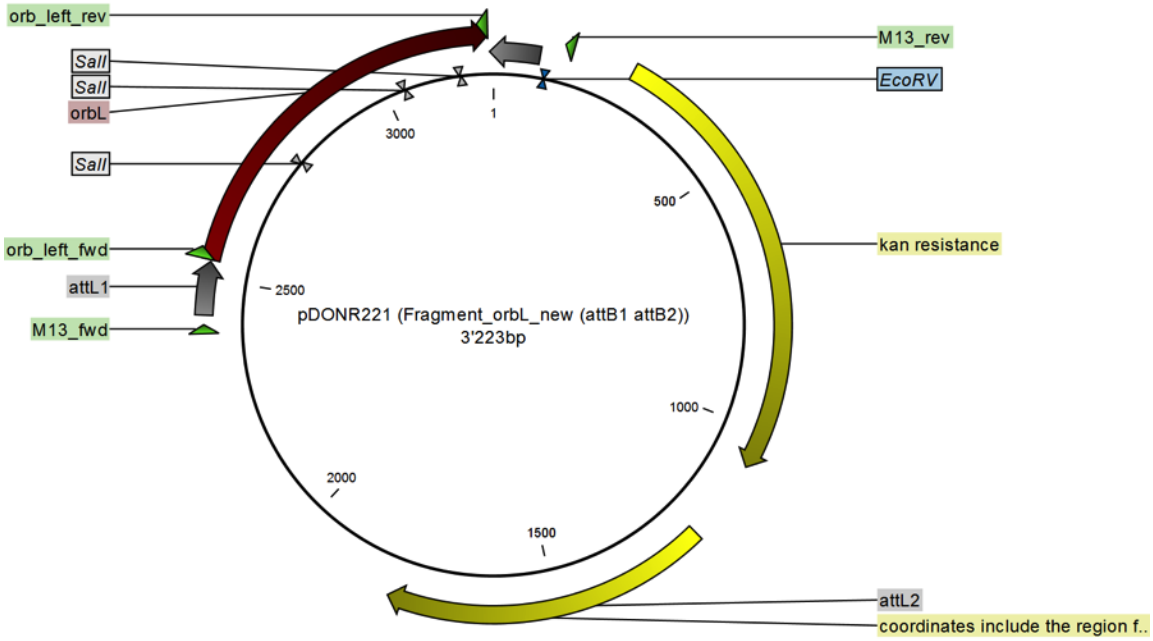
Figure 17: Construction of *B. saccahari*_Orb and *B. tuberum*_Orb by Tn7 capture system

1. Ornibactin locus (approx.35kb target locus to be transferred to *B. sacchari* and *B. tuberum*)

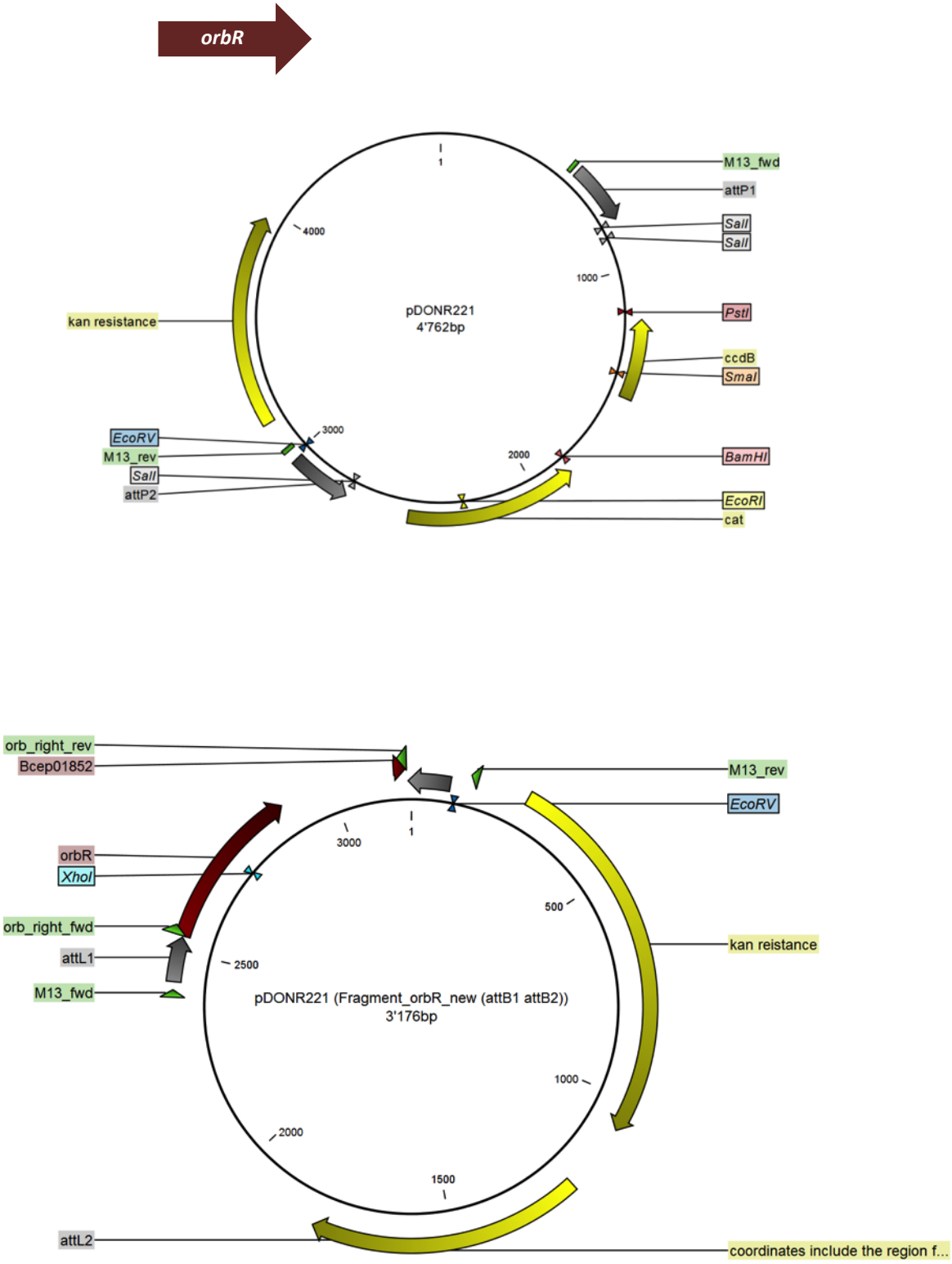


2a) The left flanking *orbL* gene cloned to the entry vector pDONR221 resulting in pDONR221::orbL

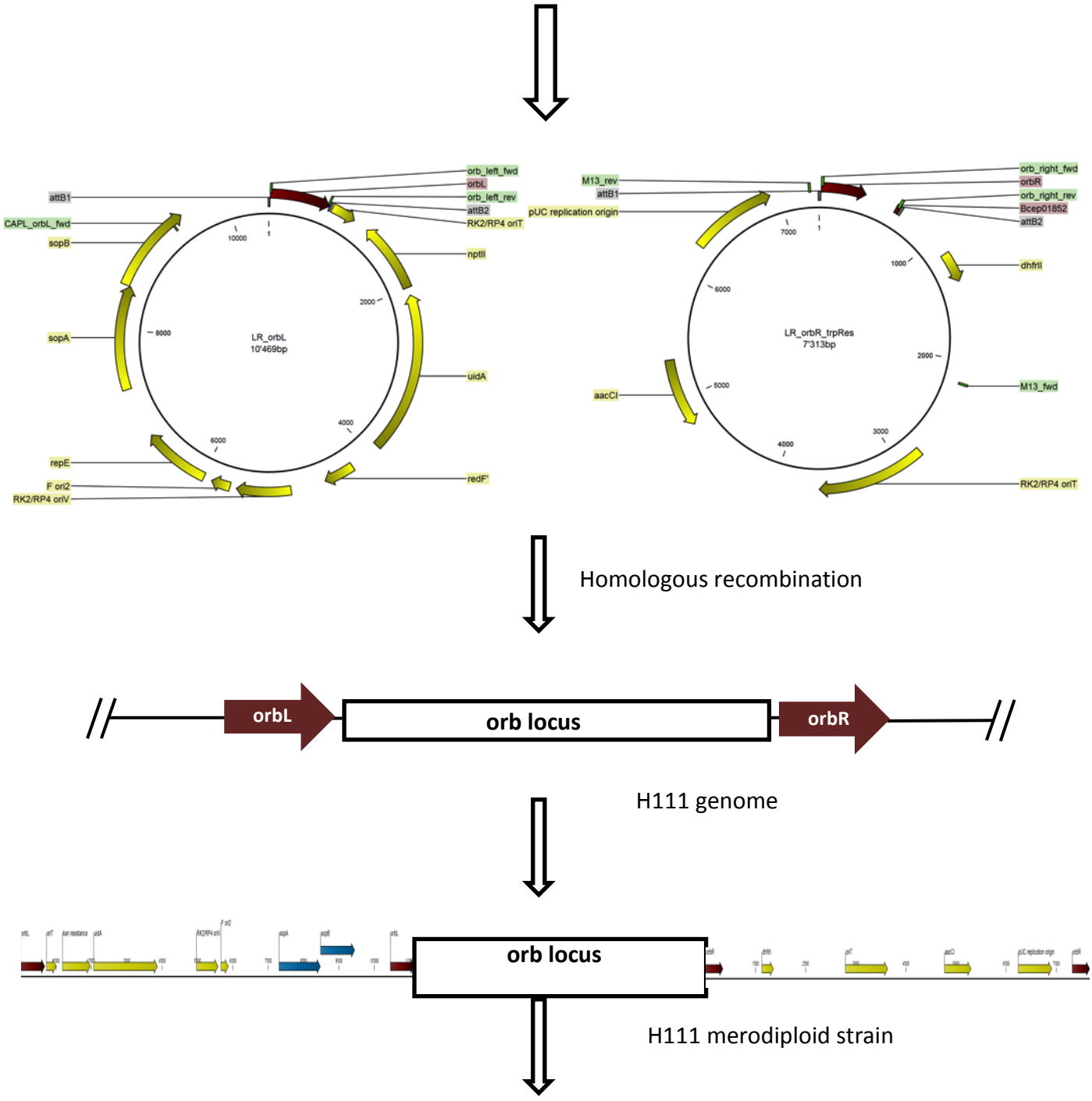




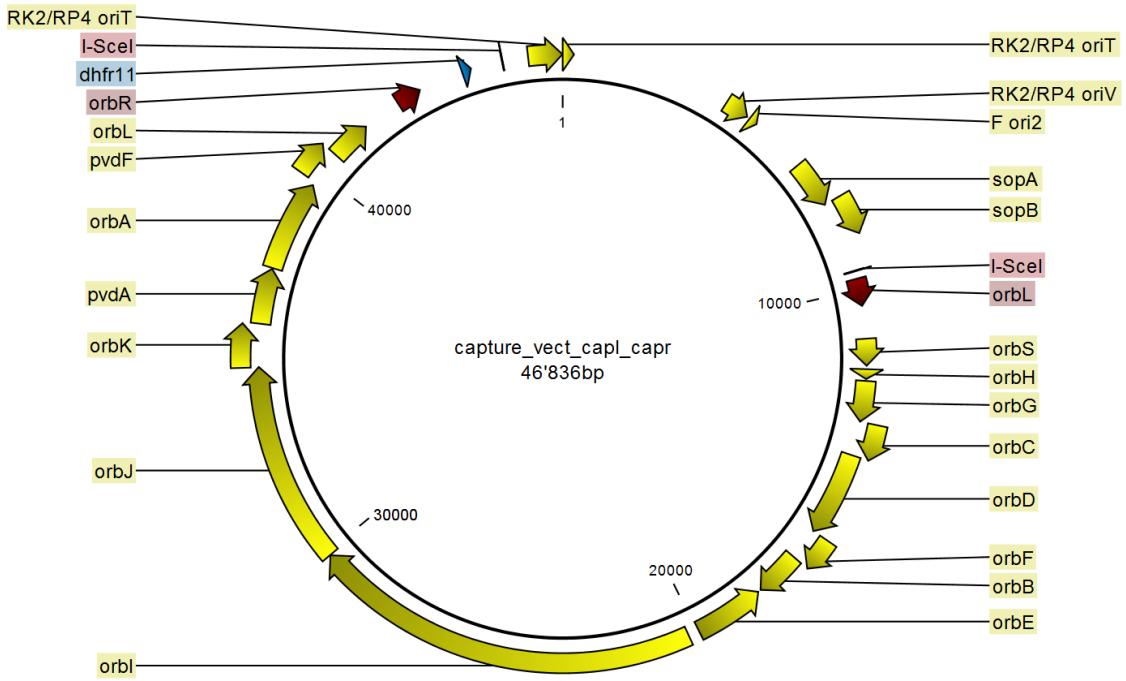
2b. The right flanking *orbR* gene cloned to the entry vector pDONR221 to construct pDONR221::*orbR*



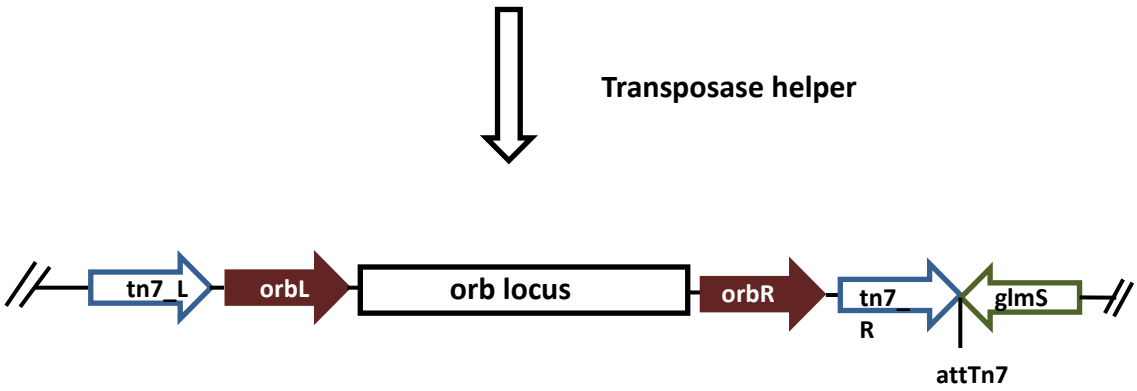
3. *orbL* and *orbR* genes subcloned into separate miniTn7 capture suicide vectors pFTCAPL and pUCTCAPR respectively and both suicide vectors are homologously recombined into *B. cenocepacia* H111 genome to generate H111 merodiploid strain



4. Conjugative transfer of ornibactin locus starting from the first oriT site and terminated at the second oriT site into a recipient *E. coli* strain, resulting in a circular pOrnibactin-Tn7 vector



5. Conjugation of the pOrnibactin-Tn7 vector with the transposase helper strain pTNS3 into *B. sacchari* and *B. tuberum* resulting in integration of the target orb locus in to the site specific attTn7 site.



B. sacchari / B. tuberum

Figure 18

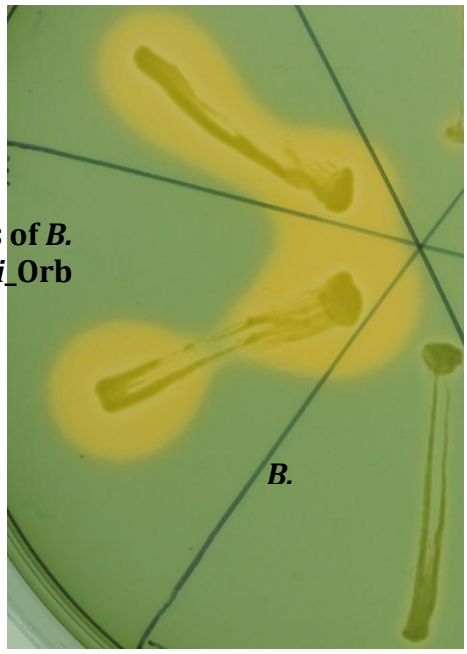


Figure 18: *B. sacchari* wild type and *B. sacchari*_Orb exhibiting ornibactin production on a CAS plate

Figure 19

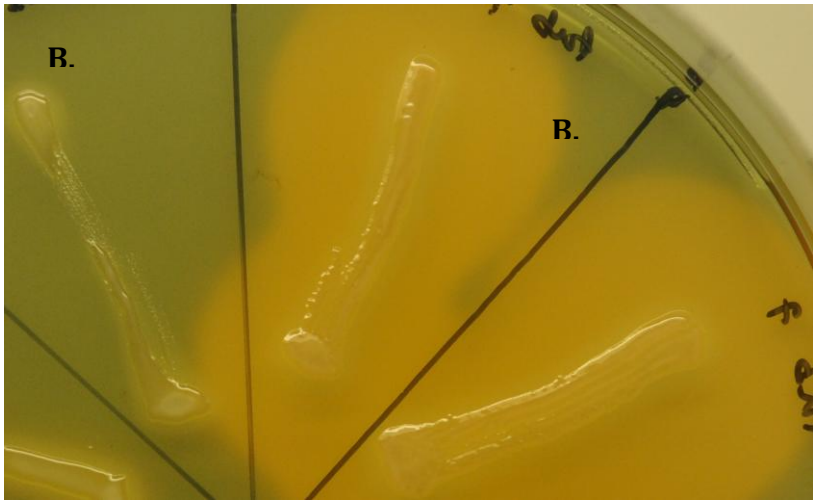


Figure 19: *B. tuberum* and *B. tuberum*_Orb displaying siderophore production in a CAS plate

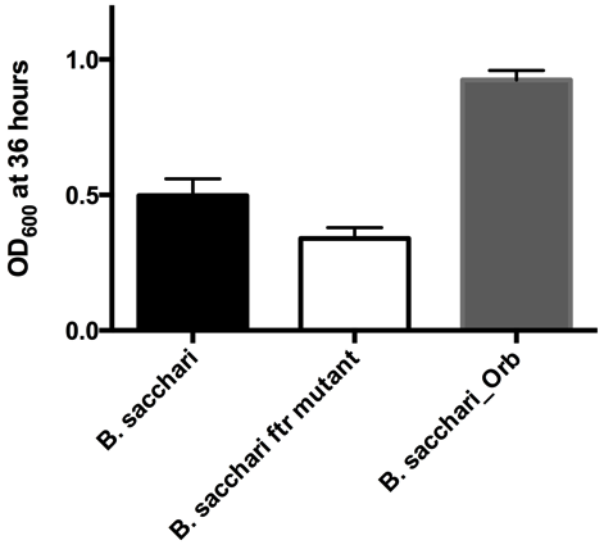


Figure 20: Growth of *B. sacchari*, *B. sacchari* ftr mutant and *B. sacchari*_Orb in M9 minimal medium in the presence of 10 µM desferoxamine

4.2.2 Production of siderophores renders *B. sacchari* pathogenic in a *G. mellonella* infection model

The ornibactin producing strains of *B. tuberum* and *B. sacchari* (which was named *B. tuberum*_Orb and *B. sacchari*_Orb respectively) were tested for virulence in a *G. mellonella* infection model. The virulence of the transgenic *B. tuberum* strain was indistinguishable from the wild type strain (Figure 21). This finding is in-line with the predicted pathogenic potential of *B. tuberum* on the basis of the analysis of its genome (Angus *et al*., 2014). However, the transgenic *B. sacchari* strain showed enhanced virulence relative to the wild type (Figure 22). The *B. sacchari* genome has not been sequenced and the presence of virulence determinants is not known in this rhizosphere isolate. We observed that *B. sacchari* does not exhibit protease activity (results not shown), which is known to be an important virulence factor of *Burkholderia*. However *B. sacchari* was able to form biofilms as assessed using 96 well microtiter plate assay, but to a lesser extent than *B. cenocepacia* H111, which was used as a positive control. Another important feature necessary to survive in a human host is the ability to grow at 37 °C. Surprisingly, *B. sacchari* is capable of growing at 37 °C, unlike most other environmental *Burkholderia* strains including *B. tuberum* (Brämer *et al*., 2001).

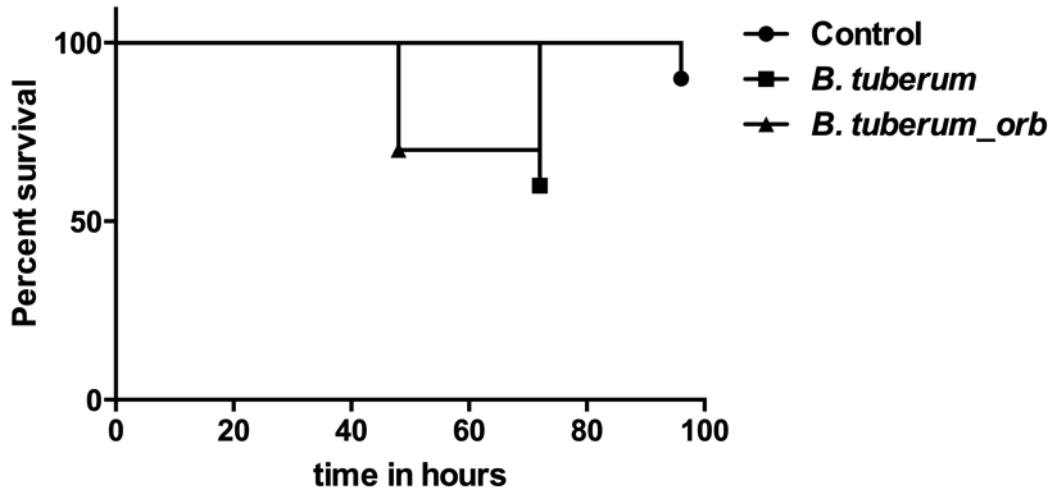


Figure 21: Pathogenicity assay of *B. tuberum* and *B. tuberum*_Orb *G. mellonella*

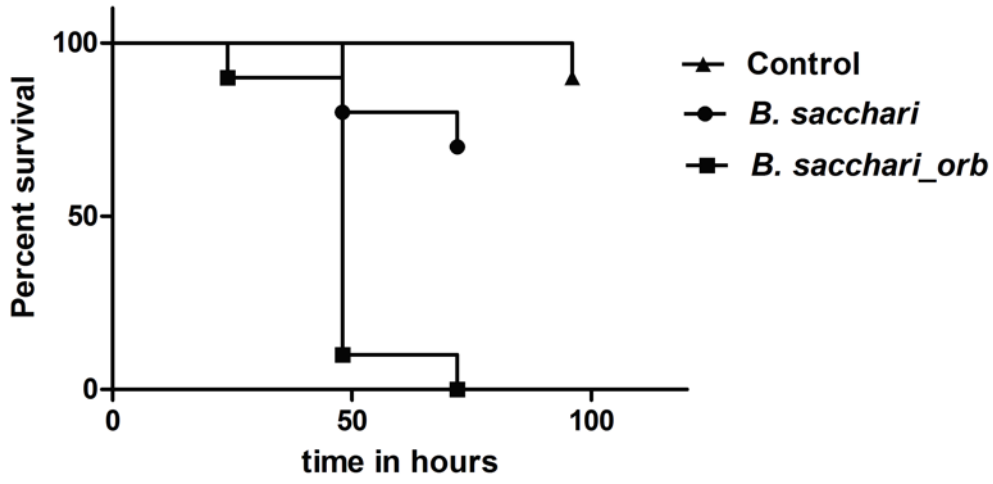


Figure 22: Pathogenicity assay of *B. sacchari* and *B. sacchari*_Orb in *G. mellonella*

4.2.3 B. sacchari producing ornibactin can grow inside *G. mellonella*

The bacterial load of the larvae infected with the different strains was determined to assess the ability of these strains to colonise and grow inside the nematode. Approximately 10^6 CFU of *B. sacchari*_Orb (*B. sacchari* harboring ornibactin locus) were recovered from the larvae after 48 hours of infection with an initial inoculum of 10^4 CFU. This shows that the strain was able to grow within the insect while the wild type *B. sacchari* strain and the two *B. tuberum* strains could not be recovered in significant numbers. This suggests that siderophores enable *B. sacchari* to grow within the infection host *G. mellonella*. (Figure 23)

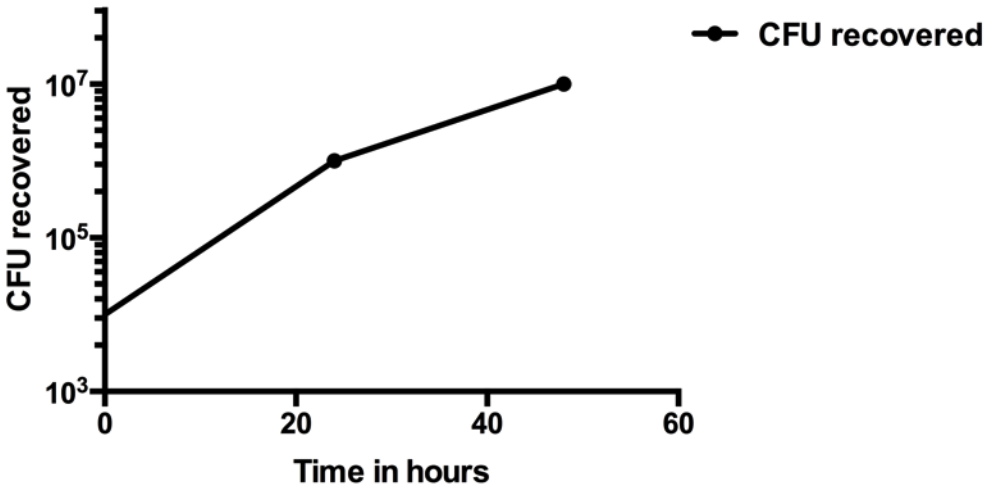


Figure 23: CFU of *B. sacchari*_Orb recovered from the hemolymph of infected *G. mellonella* larvae post infection. *G. mellonella* were infected with an initial inoculum of 10^4 CFU of *B. sacchari*. Two larvae each were crushed and homogenised in 0.5 ml PBS after 24 and 48 hours for hemolymph extraction and CFU of *B. sacchari* was enumerated by plating the strains on selective plates. Aprroximately 10^7 CFU were recovered from *G. mellonella* larvae 48 hours post infection.

4.2.4 Ornibactin does not contribute towards pathogenicity in *C. elegans*

*B. tuberum*_Orb and *B. sacchari*_Orb were also tested for pathogenicity in a *C. elegans* infection model. Production of ornibactin did not make a significant difference to the pathogenic potential of the strains towards *C. elegans* (Figure 25 and 26). This finding is in line with our previous observation that a *B. cenocepacia* H111 mutant defective in ornibactin production did not show attenuation in the *C. elegans* model (Figure 24). This is in contrast to a *B. cenocepacia* K56-2 ornibactin receptor mutant, which was previously shown to be attenuated in *C. elegans* (Uehlinger *et a*l., 2009). However, several marked differences have been reported between *B. cenocepacia* H111 and K56-2 (Uehlinger *et al*., 2009). Moreover, AidA protein, which has been shown to be important for survival and/or proliferation of *B. cenocepacia* inside the nematode intestine is critical for virulence in *C. elegans* (Huber *et al*., 2004). Environmental *Burkholderia* isolates have not been shown to produce AidA (Huber *et al.*, 2004) and a PCR analysis of *B. sacchari* for *aidA* did not give a positive result, suggesting that these strains are not able to colonise the nematode gut, probably due to the absence of the AidA protein. Bioinformatics analysis of *B. tuberum* and *B. sacchari* genome did not reveal any *aidA* homologs further confirming this finding.

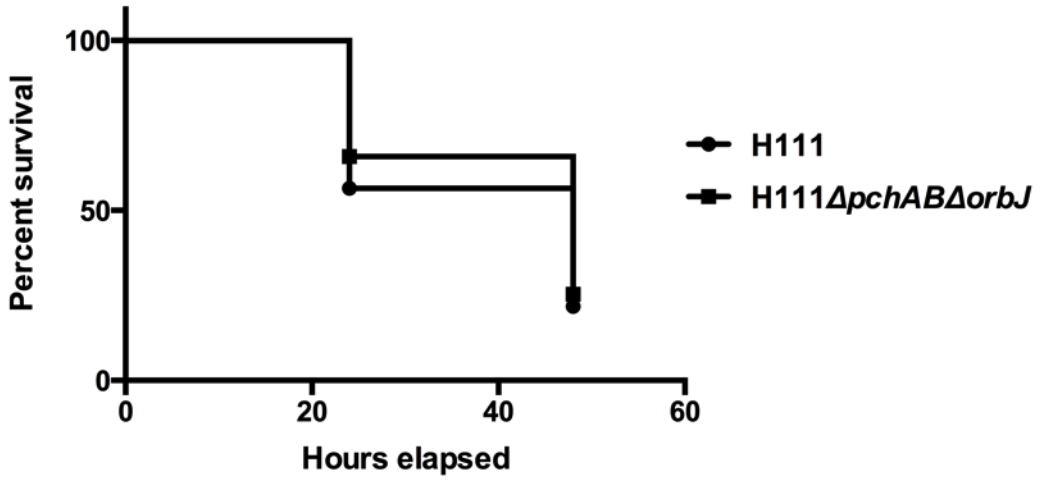


Figure 24: Pathogenicity assay of *B. cenocepacia* H111 and the siderophore mutant (H111ΔpchABΔorbJ) in C. elegans

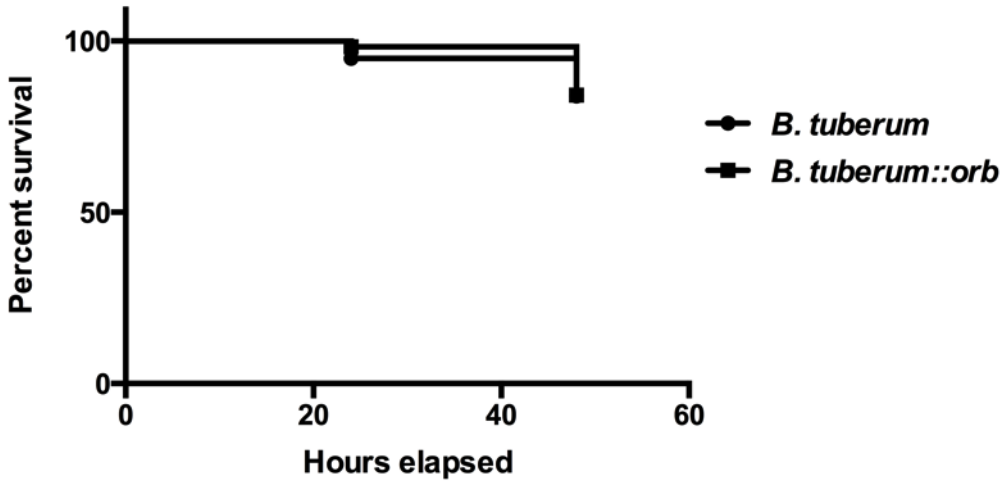


Figure 25: Pathogenicity assay of *B. tuberum* and *B. tuberum*_Orb in C. elegans

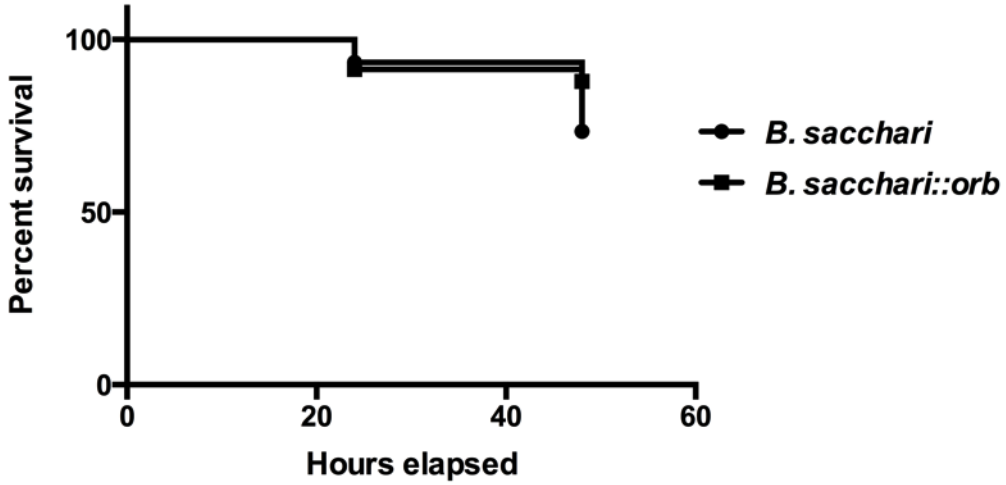


Figure 26: Pathogenicity assay of *B. sacchari* and *B. sacchari*_Orb in *C. elegans*

4.3 Discussion

Burkholderia spp. inhabit diverse ecological niches ranging from the plant rhizosphere to hospital environments (Coenye & Vandamme, 2003; Salles *et al*., 2004; Janssen, 2006). While some members of the genus are important human, animal and plant pathogens, many others are described as plant beneficial endophytes or inhabitants of the rhizosphere. Some representatives have been

shown to stimulate plant growth while others can protect plants against pathogens or improve the acquisition of essential nutrients (Compant *et al*., 2008). The beneficial effects of *Burkholderia* have stimulated interest in using these strains as plant-growth promoting and biocontrol agents. However, a clear distinction between pathogenic and beneficial strains of *Burkholderia* is currently not possible, greatly limiting the use of *Burkholderia* strains for biotechnological applications.

Burkholderia species' genomes are among the largest found in bacteria, and consist of multiple replicons (Lessie *et al*., 1996). The genomes are rich in insertion sequences, mobile elements and have a high percentage G+C content, which may contribute to the genotypic plasticity and the great strain to strain variability among *Burkholderia* isolates (Holden *et al*., 2004; Chain *et al*., 2006). This may also explain their immense adaptability to diverse environmental niches. It has been shown that conditions such as oxidative stress can stimulate movement of insertion sequences within genomes leading to genomic rearrangements and therefore survival in extreme environments (Drevinek *et al*., 2010). It was in this context that we questioned whether introduction of a key virulence determinant such as a siderophore would help *Burkholderia* adapt to different environmental conditions/habitats.

Siderophores in general, and ornibactin in particular, have been shown to be important for virulence of *B. cenocepacia* strains in different infection models (Uehlinger *et al*., 2009). We observed that *B. tuberum* and *B. sacchari* were among the few *Burkholderia* isolates that did not produce any siderophores on CAS plates (Mathew *et al*., 2014). It was particularly interesting that all the siderophore deficient strains belonged to the plant beneficial, non-pathogenic clade of *Burkholderia*. *B. tuberum* has been studied extensively due its plant beneficial symbiotic relationship with legumes. It has been shown recently that T3, T4 and T6 secretion systems commonly associated with virulent *Burkholderia* species and other virulence factors are completely absent from many plant-associated *Burkholderia* strains including *B. tuberum* (Angus *et al*., 2014). Accordingly, we observed that heterologous production of ornibactin did not increase the virulence of *B. tuberum*. However in the background of the sugarcane rhizosphere isolate *B. sacchari,*

expression of the ornibactin genes enhanced virulence. In fact, effective assimilation of iron is particularly important for bacteria to initiate infection and to survive within their hosts, where iron limitation is a major constraint imparted by the host to prevent pathogen multiplication. Consequently, pathogens are often equipped with an array of iron transporters to overcome iron deficiency. Members of the genus *Burkholderia* are also known to possess multiple iron transport strategies, which are particularly important for virulence (Visser *et al*., 2004; Uehlinger *et al*., 2009).

The complete absence of siderophores from some of the environmental *Burkholderia* isolates was unexpected, since siderophores have also been proposed to be beneficial for soil inhabitants to efficiently compete for iron. The difference between *B. tuberum* and *B. sacchari* in virulence when modified to produce ornibactin may be attributed to certain phenotypic traits of the strains. The ability to grow at 37 °C is an important feature of most pathogens (Araujo & Rodrigues, 2004) and it has been shown that *B. tuberum* is not capable of adapting to mammalian body temperatures (Angus *et al*., 2013). However, *B. sacchari* can grow well between 25 °C and 37 °C (Bramer *et al*., 2001) and its ability to survive inside the host could be partly attributed to this feature.

The virulence assays using *C. elegans* as infection host did not show any significant difference in virulence, regardless of the production of ornibactin. Previous work on the role of ornibactin in virulence showed that it was important for infection in *C. elegans* (Uehlinger *et al*., 2009). These pathogenicity assays were performed in a *B. cenocepacia* K56-2 ornibactin receptor mutant, and this strain showed attenuation in *G. mellonella*, *C. elegans* as well as in rat agar bead models. However we did not observe any significant difference in virulence between an ornibactin-deficient mutant of the related strain *B. cenocepacia* H111 and the wild type in *C. elegans*, though the ornibactin mutant was attenuated in *G. mellonella*. This difference may be attributed to strain to strain variability between the related H111 and K56-2 strains (Uehlinger *et al*., 2009). It has been shown that AidA is an important virulence factor and is critical for the persistence of *Burkholderia* strains in the *C. elegans* intestinal gut (Huber *et al*., 2004). The absence of this virulence factor in the environmental *Burkholderia* isolates explains their inability to infect *C. elegans*.

Our data show that the acquisition of a single virulence determinant can transform a harmless environmental *Burkholderia* strain into a pathogen. Although virulence was only tested in non-mammalian infection models in this study, previous work has shown ornibactin to be an important virulence factor in respiratory infections in rats (Visser *et al*., 2004). It is important to keep in mind that *Burkholderia* species are particularly abundant in the rhizosphere, which harbours a large number of other bacteria and is considered a hot spot for horizontal gene transfer (van Elsas & Bailey, 2002). Although knowledge on the extent of gene transfer in natural environments is scarce, it may be sufficient to transfer a single virulence determinant to render a strain pathogenic. Our findings suggest that the possibility of gene transfer has to be considered in the assessment of the pathogenic potential of beneficial *Burkholderia* strains in biotechnological applications.

4.4 Materials and Methods

4.4.1 Bacterial strains and media

Strains and plasmids used in this study are listed in Table S1and primers are listed in Table. S2. *B. cenocepacia* H111, an isolate from a cystic fibrosis patient (Gotschlich *et al*., 2001), B. tuberum STM 678, a legume nodule isolate (Vandamme *et al*., 2002) and *B. sacchari*, a sugarcane rhizosphere isolate (Bramer *et al*., 2001) were the important strains used in this study. *Burkholderia* strains were grown at 37°C in iron limited medium (Meyer and Abdallah, 1978) for growth assays and in Luria Bertani medium for pathogenicity assays. For E. coli cultures, antibiotics were added at the following concentrations, when required: ampicillin (Amp), 100 µg/ml; kanamycin (Km), 50 µg/ml;; trimethoprim (Tp), 50 µg/ml; chloramphenicol (Cm), 25 µg/ml; streptomycin (Strep), 50 µg/ml; gentamicin (Gm), 10 µg/ml; 15 µg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). Arabinose (ARA) was used at a final concentration of 0.2% to induce gene expression from plasmids in *Burkholderia* spp.

E. coli strains top 10 (Invitrogen), cc118 λpir (Invitrogen) and S17 were used as cloning hosts.

4.4.2. Bacterial genetics

We used the oriTn7 capture system developed for *B. pseudomallei* (Kvitko *et al*., 2013), with slight modifications, to specifically clone the ornibactin locus of around 35 kb into different *Burkholderia* strains as depicted in Figure 1. This procedure is based on site specific recombination between oriT sites of conjugative plasmids (Chain *et al*., 2000)

1. oriTn7 capture of flanking regions of the ornibactin locus: Initially, left and right flanking regions of ornibactin locus were amplified by PCR and cloned into the pDONR221 gateway donor vector. pDONR221 vector carrying flanking regions are then recombined with the right and left capture vectors constructed by Kvitko *et al* (Kvitko *et al*., 2013). pUCTCAPR-GW right capture vector was modified to replace the zeocin resistance marker with trimethoprim resistance cassette to select against *B. cenocepacia* H111 in further steps. LR recombination between the right flanking fragment and pUCTCAPR-GW1 was performed using LR Clonase II (Invitrogen life technologies) according to manufacturer's instructions. The resulting recombinant clones were selected using gentamicin and further confirmed by trimethoprim resistance as well as restriction analysis using Pst1 and BamH1.

Since the left capture vector encodes a kanamycin resistance marker similar to the entry vector pDONR221, the left flanking region including the attL sites was PCR amplified using M13fwd and M13rev primers and then recombined with the pFTCAPL vector using the LR clonase. Lucigen's BAC-optimized Replicator v2.0 E. coli cells, that contain an inducible *trfA* gene, were used as cloning host and the medium was supplemented with 0.2% arabinose for induction of replication from oriV origin in the pFTCAPL vector before plasmid purification.

1. **Construction of merodiploid donor strains**: The right and left capture vectors were transformed into E. coli S17 prior to biparental mating with B. cenocepacia H111. Homologous recombination of the right capture vector was facilitated by conjugation initially and the single merodiploid clones were selected using gentamicin and trimethoprim. The right vector captured merodiploid clone was then used as the recipient for a second conjugation to homologously recombine the left capture vector to obtain the double merodiploid clones. This second

conjugation resulted in directly oriented oriT sites flanking the ornibactin locus in H111 genome. The recombination of right and left capture vectors was confirmed by PCR using primer pairs as mentioned in the supplementary material.

2. **pOrnibactin-Tn7 vector capture mating**: In the following step, the H111 merodiploid strain harboring two oriT sites was used as a donor in a triparental mating. The new broad host range conjugation helper, pBBRK2013 (Kvitko *et al.*, 2013) facilitated the transfer of ornibactin locus into the recipient E. coli replicator strain by recognising the first oriT site flanking the ornibactin locus. The target ornibactin locus and the adjacent mini F replicon was transferred till the second oriT site, which recombined with the first oriT site, thereby generating a circular mini F replicon based plasmid containing the ornibactin locus. *E. coli* transconjugants were selected on LB plates and checked for trimethoprim, streptomycin, ampicillin as well as kanamycin resistance. Conjugants were streaked for single colonies on LB containing X-gluc and the blue colonies were further checked for kanamycin resistance as well as gentamicin sensitivity.

   To confirm the generation of pOrnibactin-Tn7 vector, PCR using multiple sets of primers along the ornibactin locus as well as modified gel eckhardt electrophoresis (Crook *et al*., 2012) was performed. For Eckhardt gel electrophoresis, overnight cultures of E. coli replicator strain harbouring the pOrnibactin-Tn7 vector was resuspended in fresh LB medium and grown to an OD600 of approximately 0.6. *E. coli* carrying 52 kb pRK2013 plasmid was used as a positive control. 150 µl of culture was washed with 500 µl 0.3% sarkosyl and resuspended in 20 µl of lysis solution. Samples were run in Eckhardt gel (1× SBE [10 mM NaOH, 1 mM EDTA, 29 mM boric acid, pH 8.0], 0.9% agarose, 0.5% SDS) in 1x SBE buffer at 100 V for 2.5 h. The gel was then stained for 30 minutes in 0.4 µg of ethidium bromide per milliliter and destained for 10 min in SBE buffer before imaging.

3. **Transposition of pOrnibactin-Tn7 vector into the genomes of target strains:** The pOrnibactin-Tn7 vector was electroporated into *E. coli* S17 and transformants were selected on LB plates supplemented with kanamycin and Trimethoprim. The transformants were also checked for gentamicin sensitivity to rule out the presence of the helper plasmid. In the following transposition step, *E. coli* S17 carrying pOrnibactin-Tn7 vector was transferred by conjugation using pTNS3 as the

transposase helper to *B. tuberum* and *B. sacchari*. Conjugants were selected on PIA plates supplemented with trimethoprim and checked for kanamycin sensistivity to rule out single cross over events. Confirmation of integration of the Tn7 transposon at the established attTn7 site located downstream of the glmS gene within different clones was verified by PCR and sequencing using the primer pairs listed in the table. Ornibactin production by *B. tuberum* and *B. sacchari* conjugants was tested with the chrome azurol S assay as well as by extracting ornibactin from these bacterial cultures, as described previously (Stephan *et al*., 1993). For ornibactin extraction, *B. sacchari* orb was grown for 40 hours in IFS medium containing 5mM ornithine. 100 ml culture supernatants were concentrated under vacuum and saturated with Nacl followed by extraction of siderophore containing residue with 1:1 ratio of chloroform:phenol. The extract was resolubilised in water by adding diethyl ether and water (8: 1 ratio) to the mixture. The aqueous phase containing ornibactin was washed 3 three times with ether and evaporated to dryness and extracted using methanol.

4.4.3.*Galleria mellonella* pathogenicity assay

Infection of *G. mellonella* larvae was performed as described previously (Seed and Dennis, 2008; Uehlinger *et al*., 2009). Briefly, *G. mellonella* in the final larval stage (purchased from Fischerei Brumann, Zürich) were stored in wood shavings at 15°C and used within 1 week. Overnight cultures of bacteria in LB broth were diluted 1:100 in 30 ml LB broth and incubated with shaking at 30°C to OD_{600} of 0.4–0.7. The bacteria were harvested by centrifugation and the pellets resuspended in 10 mM MgSO4 (Merck). Culture density was adjusted to an OD_{600} of 0.025, corresponding to 4×10^7 cfu ml^{-1}. 10 µl aliquots were injected into the *G. mellonella* larvae via the hindmost proleg using a 1 ml syringe (BD Plastipak) with a 27-gauge by 7/8-in. needle (Rose GmbH, Germany). 10 µl of $MgSO_4$ was injected in to the negative control larvae. Ten larvae were used per strain tested, and each experiment was carried out in triplicate. The infected animals were incubated in Petri dishes at 30°C in the dark. The number of dead larvae was counted every 24 hours for 7 days post infection. Larvae were considered dead when they did not respond to touch.

To analyse the ability of the different *Burkholderia* strains to proliferate inside the larvae, CFUs were determined 48 hours post infection. Larvae were crushed and dilutions were plated on to LB plates with appropriate antibiotics to quantify the bacterial load in them.

4.4.4.*Caenorhabditis elegans* slow killing assay

Nematode killing assays were performed as described by Kothe *et al*. (Köthe *et al*., 2003). Briefly, overnight cultures of strains to be tested were resuspended in fresh LB medium and were grown to a density of about 1.5 x 10^4 CFU/ml. 100 µl of suspension was plated on six-well plates containing nematode growth medium (NGM II) for slow killing assays. After 24 h of incubation at 30°C, a bacterial lawn was formed, and approximately 20 to 30 hypochlorite-synchronized L4 larvae of *C. elegans* Bristol N2 (obtained from the Caenorhabditis Genetics Centre, University of Minnesota, Minneapolis) were used to inoculate the plates. The number of worms was determined by using a Stemi SV6 microscope (Zeiss, Oberkochen, Germany). Plates were then incubated at 23°C and scored for live worms for 72 hours. All experiments were carried out at least three times, and *E. coli* OP50 was used as a negative control in the assays.

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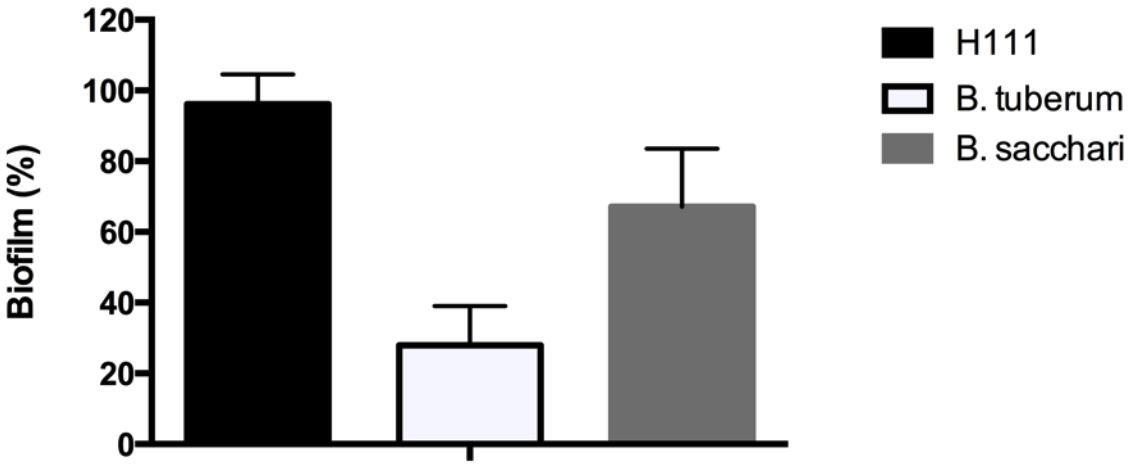

Figure 4.S1: Biofilm formation of *B. sacchari* and *B. tuberum*, in AB minimal medium, compared to *B. cenocepacia* H111. Biofilm levels are plotted as percentages compared to the positive control, *B. cenocepacia* H111. Error bars indicate values ± SEM.

Results III

Chapter 5: Results III. Role of the Ftr system and siderophores in different habitats of *Burkholderia*

5.1 Introduction

Iron is an essential cofactor for many biological processes ranging from DNA biosynthesis to oxygen transport in bacteria (Andrews *et al*., 2003). However, iron is not freely available in most habitats since it predominantly occurs in the insoluble ferric iron form or bound to host proteins such as transferrin or ferritin (Hider & Kong, 2010). Consequently, bacteria have evolved various strategies to acquire iron from diverse sources. Members of the genus *Burkholderia* are equipped with different iron transport systems, which contribute to their adaptability to various habitats ranging from the plant rhizosphere to lungs of cystic fibrosis patients (Thomas, 2007; Mathew *et al*., 2014; Coenye & Vandamme, 2003).

One of the well-known strategies for iron uptake by the majority of bacteria including *Burkholderia* is by means of siderophores. Members of the genus have been demonstrated to produce at least five different siderophores, namely pyochelin, ornibactin, cepabactin, malleobactin and cepaciachelin (Thomas, 2007). In addition, *Burkholderia* strains are also capable of utilizing hemin and ferritin as iron sources (Whitby *et al*., 2006). Recently, we reported that *Burkholderia* species possess an alternative iron transport system, FtrABCD, which is highly conserved among isolates from different niches (Mathew *et al.*, 2014). It is not unusual for bacteria to produce structurally diverse siderophores as exemplified by *E. coli*, for which they constitute an important strategy for host immune response evasion (Bachman *et al*., 2012). However, employing diverse iron transport strategies might be instrumental in acquiring iron from different environmental niches.

A role of siderophores, particularly ornibactin, in the pathogenicity of *Burkholderia* species has been well established (Visser *et al*., 2004; Uehlinger *et al*., 2009) whereas the Ftr system is dispensable for virulence (Mathew *et al*., 2014). However, the ubiquity of the Ftr system in *Burkholderia* strains, particularly in the environmental isolates, that often lack siderophores, is evidence of the importance of this novel

system in iron transport. Although the different systems may be functionally redundant to some degree, we hypothesized that the diversity of iron uptake systems in *Burkholderia* may reflect adaptation to different niches.

In a survey of siderophore production and the presence of *ftrABCD* genes among *Burkholderia* isolates, we identified 2 *Burkholderia* strains isolated from legume root nodules and 3 soil isolates that did not produce siderophores on CAS plates (Mathew *et al.*, 2014). The two nodule isolates, *B. tuberum* and *B.mimosarum,* have been shown to form an effective symbiosis with tropical legumes (Vandamme *et al*., 2002; Chen *et al*., 2006). The bacteroids involved in an active symbiosis have an increased demand for iron because many of the proteins important for the symbiosis contain iron, including nitrogenase, cytochromes and ferredoxin (Brear *et al.*, 2013). Iron transport by siderophores is proposed to be dispensable for symbiosis in rhizobia since siderophore receptors and the TonB protein were shown to be down-regulated in bacteroids (Yeoman *et al*., 2000; Small *et al*., 2009). We observed that *B. tuberum* does not produce siderophores *in vitro* on CAS plates. Genome analysis of *B. tuberum* revealed that it does not possess any known siderophore biosynthesis genes, although it encodes TonB-dependent receptors (Angus *et al*., 2013; Agnieszka Klonowska., personal communication).

Burkholderia strains are widely associated with soil and the rhizosphere. Although siderophores have been shown to be abundant in the plant rhizosphere and are proposed to be important for growth of *Burkholderia* in soil (Jurkevitch *et al*., 1992; Bishop & Rachwal, 2014), the soil isolates *B. sacchari* and *B. sartisoli* did not exhibit any siderophore activity on CAS plates. However, the Ftr system was found to be present in all environmental *Burkholderia* isolates tested (Mathew *et al*., 2014). This finding suggests that the Ftr system could be important for iron transport and growth of *Burkholderia* strains in soil.

In this study, we investigated the importance of siderophore and Ftr systems in the establishment of a symbiosis with legumes, as well as for the growth of *Burkholderia* in a soil microcosm. We show that the Ftr system has a major role in the growth of *Burkholderia* in soil microcosms, while neither of these systems seems to be essential for nodule initiation and symbiosis establishment.

5.2 Results

5.2.1 The Ftr system is unnecessary for nodulation and symbiosis of *Burkholderia* with legumes

To examine the importance of the Ftr system in nodule initiation and symbiosis, the transcript levels of *ftrA* in cells cultured in iron-limited medium and bacteroids were compared using qPCR. We observed that transcription of *ftrA* in *B. tuberum* was induced in free-living cells when compared with bacteroids (Table 6). Moreover, the siratro plants as well as the number and appearance of siratro root nodules colonized by a *B. tuberum ftrC* mutant was indistinguishable from the wild type. (Figure 27A, 27B, 28A and 28B).

Table 6: Relative expression levels of *ftrA*

Condition	Fold change	P value
Bacteroid Vs. free living *B. tuberum*	-1.73 ± 0.32	0.0001

Figure 27A

Figure 27B

Figure 27: (A) Siratro plants infected with *B. tuberum* and (B) plants infected with an *ftr* mutant of *B. tuberum*

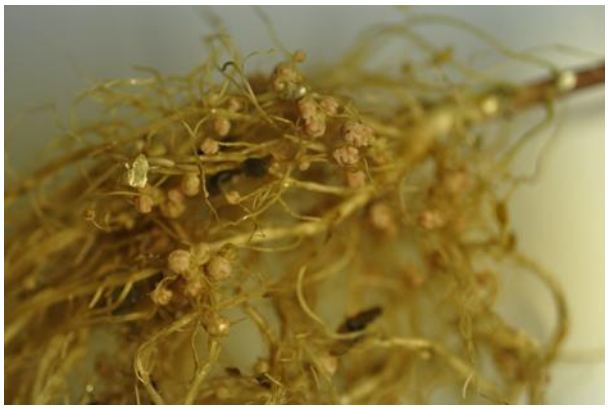

Figure 28: (A) Nodules infected with *B. tuberum* and (B) Nodules infected with an ftr mutant of *B. tuberum*

5.2.2 Ornibactin production does not increase the efficiency of nodulation of legumes by *B. tuberum*

It has been previously shown that *Sinorhizobium meliloti* is more efficient in nodule occupancy compared to mutant strains with impaired siderophore uptake systems (Battistoni *et al*., 2002). We therefore transferred the ornibactin locus encoding ornibactin synthesis and transport genes in to *B. tuberum* and analysed whether production of ornibactin increased the symbiotic efficiency of the strain. Plants infected with the *B. tuberum*_Orb mutant strain displayed similar growth and nodule development as plants inoculated with the wild type. No significant difference in nodule number or pattern was observed. The number of bacteria, which could be re-isolated from the nodules, showed similar viable counts (Figure 29). These data suggest that ornibactin production does not increase the nodulation efficiency.

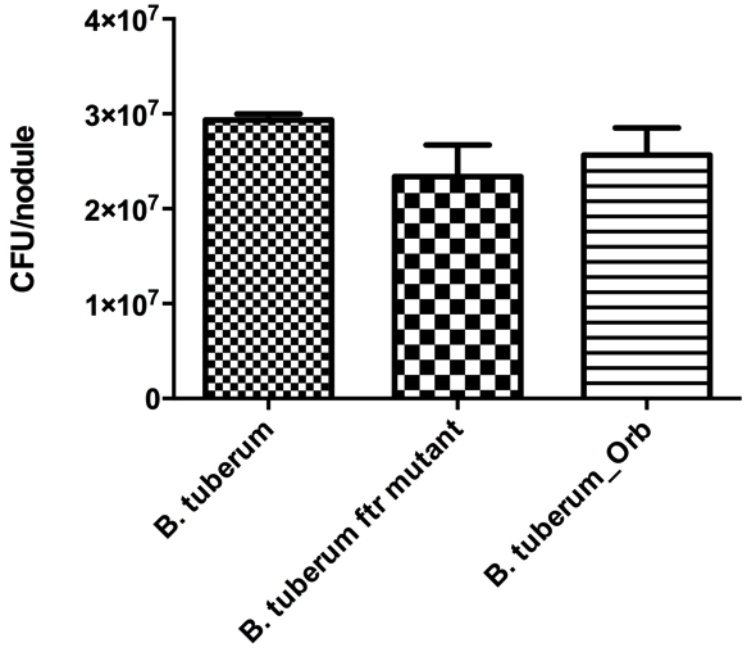

Figure 29: CFU counts of *B. tuberum* ftr mutant and *B. tuberum*_Orb isolated from nodules

5.2.3 Ornibactin provides a competitive advantage for nodule occupancy

It has been reported that siderophores provide a competitive advantage to rhizobia over non-producers in nodule occupancy as well as symbiosis (Battistoni *et al*., 2002; Brear *et al*., 2013). To investigate whether production of ornibactin provides a competitive nodulation advantage to *B. tuberum* a mixture containing equal amounts of the *B. tuberum* wild type and *B. tuberum*_Orb was used to infect siratro seedlings. All nodules from each plant were collected 28 dpi and the bacteria were isolated from the nodules. The wild type to *B. tuberum*_Orb ratios in the nodules was determined. As shown in figure 30, *B. tuberum*_Orb was more efficient in occupying nodules compared to the wild type. The *ftrC* mutant of *B. tuberum* was virtually indistinguishable in nodule occupancy from the wild type. These findings suggest that siderophore mediated iron transport provides the strain a competitive advantage and that the Ftr system is not important for *B. tuberum* to establish a symbiosis with legumes

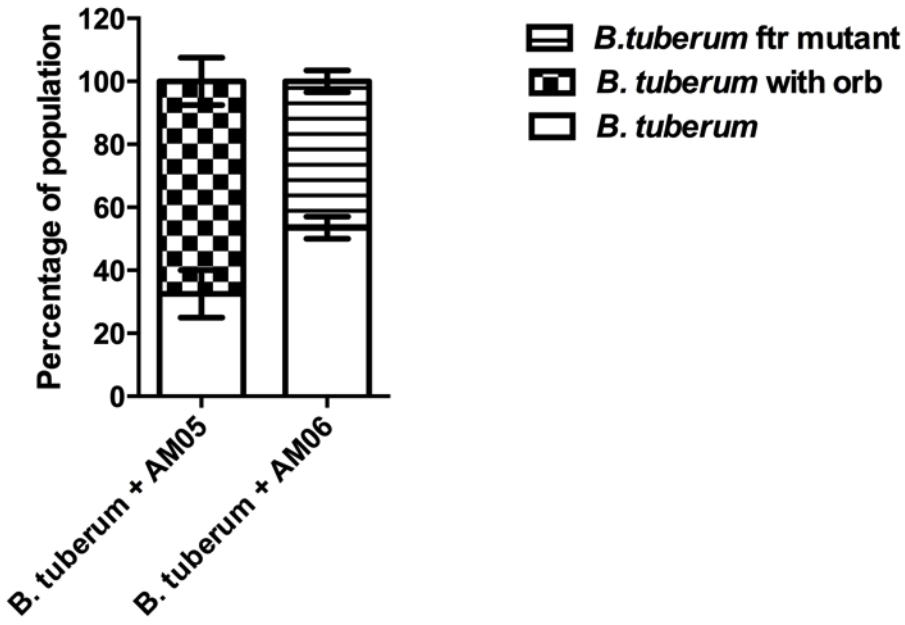

Figure 30: Competition between the *B. tuberum* wild type and an isogenic *ftrC* mutant as well as a recombinant *B. tuberum* strain producing ornibactin for occupancy of nodules. Siratro seedlings were co-inoculated with *B. tuberum* and *B. tuberum*_Orb or *B. tuberum* and the *B. tuberum ftr* mutant in equal amounts. Percentages of wild type and mutant strains recovered from plant nodules at different inoculum ratios were compared (not shown). Bars represent mean values ± standard errors of percentages of bacteria recovered from crushed nodules of three plants. Statistical analyses were performed using Prism graphpad.

5.2.4 The Ftr system is important for growth of *Burkholderia* in a soil microcosm

Considering the prevalence of *Burkholderia* in the plant rhizosphere and soil, as well as the absence of siderophore production in some of the soil *Burkholderia* isolates, we investigated the importance of the Ftr and siderophore systems for proliferation in soil. Soil collected from an agricultural field in Aberdeen, from which *Burkholderia* species had been previously isolated (Stopnisek *et al*., 2014) was used for this experiment. We tested survival and growth of the *B. cenocepacia* H111 wild type, the ornibactin and pyochelin double mutant BccAM03, and the *ftr* mutant BccAM04 in soil. As depicted in figure 31, growth and persistence of the siderophore null mutant BccAM03 was indistinguishable from the wild type, indicating that siderophore-mediated iron transport is not essential for soil colonization. However, the Ftr mutant BccAM04 showed reduced survival in the microcosm, demonstrating the importance of this iron transporter for growth and persistence in soil. We were unable to recover strain BccAM05, lacking functional siderophores and the Ftr iron transport systems, from soil after 1 day of incubation. This result confirms that iron (III) uptake is essential for survival in soil.

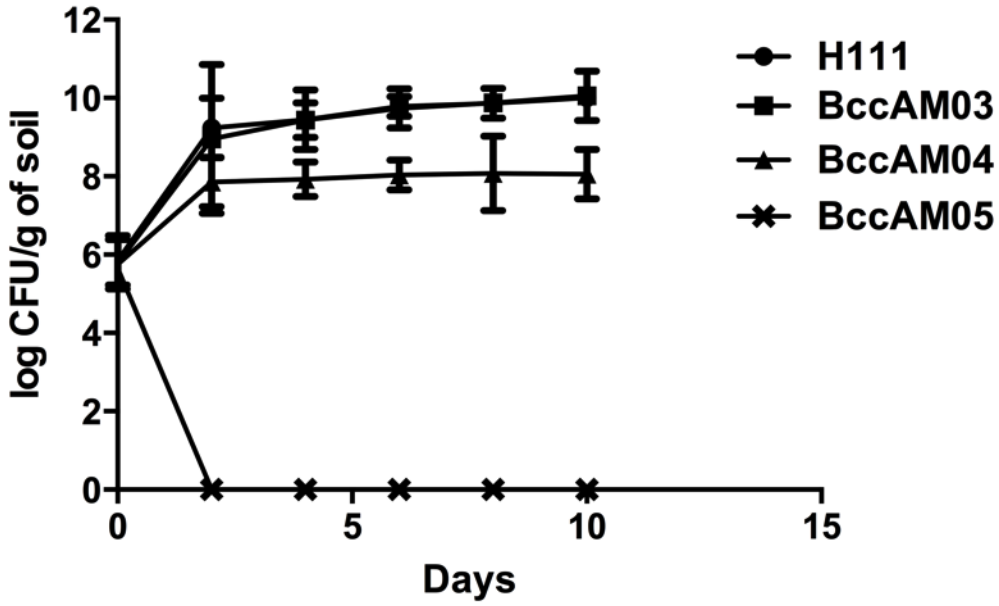

Figure 31: Survival of *B. cenocepacia* H111 and different mutants in a soil microcosm. The microcosm was inoculated with 6 X 10^8 bacteria per gram of soil and bacterial counts were determined over a period of 10 days. Data represent mean ± S.E of three independent experiments

The experiment was repeated using two other soil samples collected from a grassland site at Agroscope Reckenholz-Trinikon research station, Switzerland, and the Botanical garden, Zurich. While the microcosm experiment using Agroscope soil showed similar results to those obtained for the Aberdeen soil, no significant difference between colonization levels of wild type *Burkholderia* strains and mutants were observed in the soil from the Botanical Garden. This discrepancy could be due to differences in the soil parameters, such as different metal concentrations and clay content. This could not be verified since the soil parameters were evaluated only for the soil sample from Aberdeen.

Previous reports have shown that siderophores can get adsorbed on metal oxides present in many environments (Upritchard *et al.*, 2007). We hypothesized that the siderophores produced by *B. cenocepacia* H111 were adsorbed on metal oxides or montmorillonite making it a less efficient strategy for iron transport in soil. To test this hypothesis, ornibactin and pyochelin extracted from H111 was added to the soil microcosms and extracted the dissolved and adsorbed fraction of these siderophores 10 days after addition. Both extracted fractions were plated onto a CAS plate to determine the amount of siderophores. This analysis showed that pyochelin and ornibactin are mainly adsorbed to particles and only small amounts of the siderophores are in the dissolved fraction (Figure 32). This suggests that a large portion of siderophores is tightly bound to soil components and is therefore unavailable for the producer.

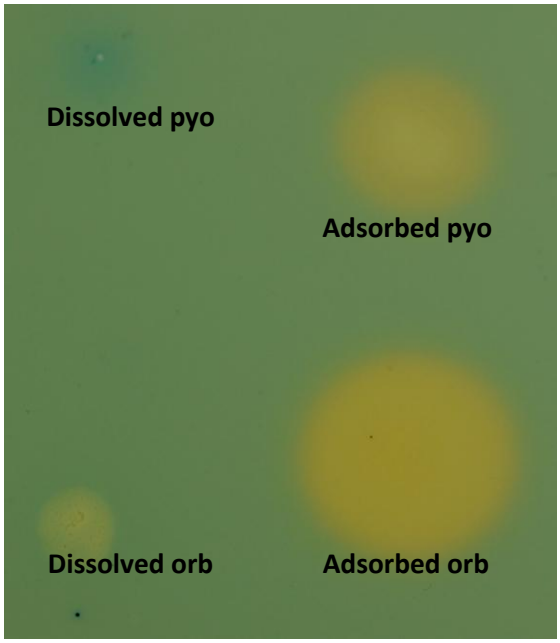

Figure 32: CAS plate displaying dissolved and adsorbed fraction of pyochelin and ornibactin from soil microcosms. Dissolved and adsorbed fractions were extracted separately from the soil microcosm after 10 days of inoculation and the fractions were spotted onto CAS plates

5.3 Discussion

Since members of the genus *Burkholderia* live under diverse environmental conditions, it is not surprising that they possess multiple iron transport strategies to acquire this micronutrient metal in their different habitats. In this study, we investigated the importance of two iron transport systems in *Burkholderia* in two different habitats and demonstrated nonequivalent roles of siderophores and the Ftr system in colonization of these habitats.

Members of the genus *Burkholderia* are among the most effective symbionts of legumes among ß-rhizobia (Gyaneshwar *et al.*, 2011). Both legumes and their symbiotic bacteria have a higher requirement for iron than their free-living counterparts since many of the proteins that are essential for the symbiosis contain this metal. In line with the increased requirement, nodules of soybean plants have been reported to have a high proportion of iron compared to other plant parts (Burton *et al*., 2008). Although *Burkholderia* species are known to employ various iron

transport systems, the mechanism used by *Burkholderia* during symbiosis is not known. We observed that neither siderophores, nor the Ftr system, are essential for nodule occupancy or symbiosis. It could be that there is an alternative, as yet unknown bacteroid iron transport system. Siderophores produced by *Rhizobium leguminosarum* were shown not to be required for iron transport into bacteroids (Yeoman *et al*., 2000). Moreover, it has been reported that bacteroids of *B. japonicum* transport iron in the reduced ferrous form more efficiently than in the ferric form (Moreau *et al*., 1998). *B. japonicum* bacteroids were also shown to transport iron complexed with another ferric chelator, citrate (Moreau *et al*., 1995), though the transport appears to be slower than with ferrous iron. Hence, the dispensability of both the siderophores and the Ftr system for the symbiosis of *Burkholderia* is not too surprising considering that they prefer ferrous iron.

We observed that addition of ferric citrate to the medium stimulated the growth of *B. tuberum in vitro* in iron-limited conditions. This observation suggests that this could be an effective iron transport strategy employed by *Burkholderia* bacteroids. Specific ferrous iron transport systems have not yet been described in *Burkholderia* and it is therefore not possible to investigate the relative importance of the two iron forms in nodule development and symbiosis. However, siderophore-mediated iron transport appears to provide a competitive advantage to the producers and may contribute to effective symbiosis of *B. tuberum*. Although ferric iron chelates are not the major iron form taken up by bacteroids, iron has been shown to be stored in the ferric form in symbiosome space as siderophore iron complexes (LeVier *et al*., 1996). Ferric reductase activity has also been detected across the peribacteroid membrane suggesting that ferric iron is reduced to ferrous iron before transport (LeVier *et al*., 1996). This observation partly explains the competitive advantage provided by siderophores in the symbiosis of *B. tuberum*.

Members of the genus *Burkholderia* are also predominantly associated with complex environments such as soil or rhizosphere soil (Suárez-Moreno *et al*., 2012). Iron availability in soil is an important parameter for the growth and survival of bacteria. In support of this view, it has been shown that siderophores play a crucial role in the survival of *B. thailandensis* in soil (Bishop & Rachwal, 2014). In addition, siderophore production is also considered a beneficial trait in soil bacteria, as these molecules can

suppress the growth of plant pathogens by competing for iron (Lugtenberg & Kamilova, 2009). We identified three soil and rhizosphere isolates of the genus *Burkholderia* that were defective in siderophore production but possessed the Ftr system, suggesting that siderophores may not be required for the survival of these strains in soil. Our soil microcosm experiments using *B. cenocepacia* H111 and isogenic mutants defective in siderophore production or the Ftr system revealed that siderophore production may not always be a sensible strategy for iron acquisition in certain soils, where siderophores are lost due to diffusion or retained by adsorption to soil particles. In contrast, the siderophore independent Ftr transport system appears to be instrumental in the uptake of iron by these strains and is therefore important for the growth and survival of *Burkholderia* in soil.

In line with our hypothesis, siderophores were adsorbed on soil particles in our microcosm experiment. Indeed, it has been shown previously that hydroxamate siderophores are irreversibly adsorbed onto clay or montmorillonite which are predominantly found in soils (Siebner-Freibach *et al*., 2004). This could also potentially impair the iron binding property of the chelator. Siebner-Freibach *et al*. also observed that the intramolecular hydrogen bonds of the ligand were weakened upon adsorption to soil particles, which could further affect stability and function (Siebner-Freibach *et al*., 2006). Our results indicate that siderophores in *Burkholderia* may also be irreversibly adsorbed on soil components. We performed experiments to analyse the adsorption mechanism of *Burkholderia* siderophores in a defined artificial soil microcosm to confirm the irreversible binding of the siderophores. However the *B. cenocepacia* strains did not exhibit consistent growth in the artificial soil medium, which had been shown to support the growth of some *Burkholderiaceae* species (Ding *et al*., 2013).

While siderophores and the Ftr system seem to be partially redundant in their function in *Burkholderia* sp., our study demonstrates that depending on the environment each of the systems is of particular importance. Previous work and our findings have confirmed the significance of *Burkholderia* siderophores in pathogenicity and metal homeostasis (Uehlinger *et al*., 2009; Mathew *et al*., 2014; our unpublished observations). Here, we show that siderophores give a competitive advantage to nodulating *Burkholderia* strains when establishing a symbiosis.

However, for the active symbiosis neither siderophores nor the Ftr system is required, suggesting that another, as yet unidentified iron transport system is important for inhabiting nodules. Further work will be required to identify this alternative iron transport mechanism as well as to confirm the irreversible adsorption of siderophores to soil components.

5.4 Materials and methods

5.4.1 Bacterial strains and growth media

Strains and plasmids used in this study are listed in table S1. Wild type *B. cenocepacia* H111 and the mutants BccAM03, BccAM04 and BccAM05 were the strains used in the soil microcosm study. The construction of these mutants was described previously (Mathew et al., 2014). *B. tuberum* STM 678, *B. tuberum ftr* mutant as well as *B. tuberum* harbouring the ornibactin locus (*B. tuberum*_Orb) were used in nodulation experiments. The introduction of the ornibactin gene cluster into *Burkholderia* strains is described elsewhere in this thesis (Page no.X) and construction of the *B. tuberum ftr* mutant is described below. The strains were either grown in Luria Bertani medium or iron limited succinate medium (Meyer and Abdallah, 1978). When required, the antibiotic trimethoprim was added at a concentration of 50 μg ml^{-1}.

5.4.2 Construction of a *B. tuberum ftr* mutant

To generate a rhamnose-dependent conditional mutant of *B. tuberum*, a 300 bp fragment of $ftr_{Bcc}C$ starting from the start codon was amplified and ligated into the vector pSC200 (Ortega *et al*., 2007). The resulting recombinant plasmid, in which a rhamnose-inducible promoter controls expression of *ftrBccC*, was transformed into *E. coli* cc118λpir. It was subsequently transferred to wild type *B. tuberum* by triparental mating. Homologous recombination resulted in insertion of a rhamnose-inducible promoter directly upstream of $ftr_{Bcc}C$. The resulting conditional mutant, designated *B. tuberum* AM01 was selected on Pseudomonas Isolation Agar (PIA) medium supplemented with trimethoprim (50 μg ml^{-1}).

5.4.3.Analysis of transcriptional regulation of $Ftr_{Bcc}ABCD$ by quantitative Real-Time qPCR

B. tuberum cells were grown to mid-exponential phase in IFS medium, RNA was extracted as previously described (Pessi et al., 2007) and further purified using RNeasy Qiagen kit.

First strand cDNA was synthesized using random primers (Invitrogen) and MLV reverse transcriptase (Promega). qPCR was performed on the cDNA using Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent, Switzerland) and a Mx3000P instrument (Agilent, Switzerland). Primers were generated using Primer 3 software (Rozen & Skaletsky, 1998)and are listed in Table 8.1. Each reaction was run in triplicates and melting curve data was analysed to determine the PCR specificity. The *recA* gene was used as the reference gene for normalization. Relative expression levels of the target gene, $ftr_{Bcc}A$ were calculated as previously described (Pfaffl, 2001). cDNA of *B. tuberum* bacteroids was kindly provided by Martina Lardi.

5.4.4.Nodulation experiments and re-isolation of strains from nodules

Sterilization of siratro (*Macroptilium atropurpureum*) seeds was done as described earlier (Koch *et al*., 2010). Sterilised seeds were then inoculated with overnight cultures of *B. tuberum* and other strains that had been diluted to approximately 1000 bacteria per seed. Nodules were collected after 28 days post inoculation; bacteria re-isolated from randomly selected nodules and selected using appropriate genetic markers.

5.4.5.Competition symbiosis experiments

Competition experiments were performed as previously described (Koch *et al*., 2014) with slight modifications. Siratro seeds were infected with a mixture of the wild type, *B. tuberum* AM01 or AM02 *B. tuberum* strains in a ratio of 50:50. Overnight cultures of the wild type and other strains were diluted to the same CFU/ml and approximately 100 cells were present in the initial inoculum. Serial dilutions of the mixed inoculum were plated on selective medium to confirm the number of inoculated cells. All nodules from one plant were harvested at 31 dpi. Nodules were surface sterilized (100 % chloroform for 5 min) and rinsed 5 times in sterile distilled water. Nodules were then crushed in 2 ml saline using a mortar, and serial dilutions of this nodule suspension were spotted on plates containing the appropriate selection for strain differentiation. The plates were incubated at 28 °C for 2 days and the ratio of the mutant to wild-type bacteria in nodule extracts was determined and compared to the initial inoculum ratio. Three independent plants were processed per host and the nodule extracts were spotted in three dilutions and in spots. Data were evaluated for statistical significance using Student's *t* test with Prism graphpad software.

5.4.6.Soil sample and microcosm experiment

Soil for preparing microcosms was collected from an agricultural field in Aberdeen, and kindly provided by Nejc Stopnisek. The soil sample was autoclaved thrice at 121 °C, 15 psi for 15 minutes to sterilize, air-dried for 7 days and sieved to < 2 mm. The sieved soil was kept in a sealed plastic bag and stored at 4 °C until use. Physicochemical characteristics of the soil sample, including soil texture, pH, electrical conductivity (EC), moisture content (MC), water holding capacity (WHC), organic matter (OM), organic carbon (OC), metal content have been given in table S2. Two additional soil samples collected from a grassland site at Agroscope Reckenholz-Trinikon research station Switzerland as well as Botanical garden, Zurich were also used in the study.

For microcosm experiments, 10 g soil was placed in petri dishes and the initial moisture content was adjusted to 60 % of total water holding capacity using milliQ water. 100 µl of inoculum (10^8 CFU/mL) was dropped over the soil in each plate and the cells were spread evenly. The plates were covered with aluminium foil to maintain soil moisture during incubation and incubated at 25 °C. To maintain the soil moisture content during the experiments, weight loss was checked every 2 days and deionized water was added to replenish the moisture content. The number of *B. cenocepacia* wt and mutant bacteria (CFUs) in the soil in each plate was enumerated every second day for a period of 10 days. Triplicate soil microcosms were prepared for each strain.

5.4.7.Extraction of dissolved and adsorbed siderophores from soil

The adsorbed and dissolved siderophore fraction were extracted as previously described with some modifications (Ahmed & Holmström, 2014). For extraction of dissolved siderophores, 1 g of air dried soil sample was added to 10 ml of Milli Q- water and shaken vigorously for 2 hours. The soil solutions were then filtered through 0.45 µm filters (Millipore, Switzerland). The water extracts were pre-concentrated by rotary evaporation and the yellow-white sediment remaining was dissolved in 1 ml of milli Q- water.

For extraction of adsorbed siderophores, 1 g of air dried soil sample was added to 10 ml of methanol and shaken for 2 hours. The soil solutions were then filtered through 0.45 µm filters and methanol filtrates were evaporated using rotary evaporation. The resulting residues were dissolved in 1 ml milli-Q water.

The resulting adsorbed and extracted fractions along with the initial siderophore inoculum was spotted onto CAS plates (Schwyn et al., 1987) to assess the siderophore contents.

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6. Results IV

Chapter 6. Results IV: The role of *Burkholderia* siderophores in metal homeostasis

6.1 Introduction

It is well established that a substantial portion of enzymes require metals as their co-factor (Andreini *et al*., 2008; Waldron *et al*., 2009). Most of these enzymes are essential for biologically vital processes such as respiration, photosynthesis, replication, transcription, translation, signal transduction, and cell division and their catalytic activity depends on the presence of transition metals (Klein and Lewinson, 2011). While metals such as magnesium, calcium, zinc and magnesium are involved in substrate activation and electrostatic stabilisation, iron, copper, cobalt and molybdenum function as redox centres in different enzymes (Nies, 1999). However transition metals such as cadmium, lead, arsenic or mercury do not have any biological function and are indeed harmful to bacteria. Moreover, most transition metals are toxic at high intracellular concentrations as they lead to the production of reactive oxygen species and free radicals resulting in cell damage (Valko *et al.*, 2005). Therefore, bacteria have evolved various mechanisms to tightly regulate the intracellular concentration of these metals (Porcheron *et al.*, 2013). These mechanisms include specific transport systems, complexation by metal binding proteins or metal ion reduction.

Siderophores are low molecular weight binding proteins produced by organisms to effectively chelate iron (Neilands, 1995). Since iron is limited in many niches inhabited by microbes, siderophores are important for iron transport and therefore have been shown to be important for the growth of many organisms. However, it has been reported that siderophores can bind to metals other then iron. For example, the hydroxamate siderophore, desferrioxamine, can bind metals such as aluminium, although with less affinity than iron (Evers *et al.*, 1989). More recently, the siderophores produced by *P. aeruginosa*, pyochelin and pyoverdin, have been shown to effectively bind 16 different metals in various affinities (Braud *et al.*, 2010). Heavy metals are known to enter bacterial cells by diffusion. Hence sequestration of these metals by siderophores in the extracellular medium, thus preventing their

entry in to the cell, could be an efficient strategy employed by bacteria to maintain metal homeostasis (Schalk *et al.*, 2011).

B. cenocepacia also employs siderophores as one of its most important strategies to acquire iron. Most of the *Burkholderia* strains secrete two siderophores, pyochelin and ornibactin (Thomas, 2007). Pyochelin occurs in two stereoisomeric forms, pyochelin I and pyochelin II with 2- hydroxyphenyl-thiazoline/-oxazoline and thiazolidine-carboxylate moieties (Thomas, 2007). Ornibactin is a linear tetrapeptide derivative bearing two hydroxamate and a α- hydroxycarboxylate metal chelation group. While pyochelin is produced by *P. aeruginosa* and *P. fluorescens* (Cox and Graham, 1979; Sokol, 1984) in addition to *Burkholderia* species, ornibactin does not seem to be produced by other bacteria. Siderophores have been demonstrated to be important for pathogenicity of *Burkholderia*. However a screen of *Burkholderia* isolates from different niches and environments revealed that siderophores are one of the common traits shared by many *Burkholderia* strains, irrespective of the habitats they were isolated from (Mathew *et al.*, 2014). Moreover, *Burkholderia* strains possess an alternative iron transport system, which seems to be important for iron uptake in soil (Chapter 3 of this thesis). These findings suggest that *Burkholderia* siderophores may possess an alternative or additional role in the environmental *Burkholderia* strains that might explain the advantages of having multiple siderophores/iron transport systems.

Although pyochelin is known to bind metals other than iron, the affinity of ornibactin to other metals is not known and has not been analysed. Considering the potential additional role of siderophores produced by *Burkholderia* in metal homeostasis, we decided to investigate the importance of pyochelin and ornibactin in protecting the cells from influx of heavy metals. In this study, we show that pyochelin and ornibactin in H111 can bind to several metals other than iron. In addition, supplementation of siderophore-deficient *Burkholderia* strains with externally supplied siderophores protects these strains from the toxic effects of heavy metals. Moreover, metals such as copper induce the production of ornibactin, which indicates the importance of this siderophore in copper tolerance of the *Burkholderia* strains.

6.2 Results

6.2.1 H111 producing siderophores can tolerate toxic metals better than non-producers.

To investigate the role of siderophores in resistance to metal toxicity, the *B. cenocepacia* wild type strain H111 and its mutants deficient in either pyochelin (AM01) or ornibactin production (AM02) or deficient in both siderophores (AM03) were grown in the presence of different metals in different concentrations. Aluminium, zinc, cobalt, copper, cadmium, and lead at concentrations of 10 and 50 µM did not affect growth of the wild type strain. However, silver efficiently inhibited growth at a concentration of 10 µM and was therefore not further investigated. Aluminium was toxic at 10 µM to the double mutant AM03 (H111Δ*pchAB*Δ*orbJ*) but not to the single mutants, suggesting that both pyochelin and ornibactin have a protective effect against this metal (Figure 33A). Ornibactin protected the strain against zinc and lead at 50 and 10 µM, respectively (Figure 33B and 33C). Ornibactin was also effective in neutralizing copper toxicity (Figure 33D). The protective effect of siderophores was not diminished in the presence of excess iron in the medium, ruling out that the growth defect of the siderophore mutants is due to iron limitation (Figure 33A and 33D). Neither of the siderophores provided increased resistance against cobalt or cadmium at the concentrations tested.

Supplementation of the medium with equal amounts of pyochelin and ornibactin extracted from H111 supernatants reverted the toxic effect, confirming the protective role of siderophores in *Burkholderia* against heavy metal toxicity. This protective effect was also evident in the presence of high levels of iron in the medium, suggesting that the improved growth is due to the increased resistance to heavy metals and not due to enhanced iron assimilation in the presence of siderophores.

Figure 33A

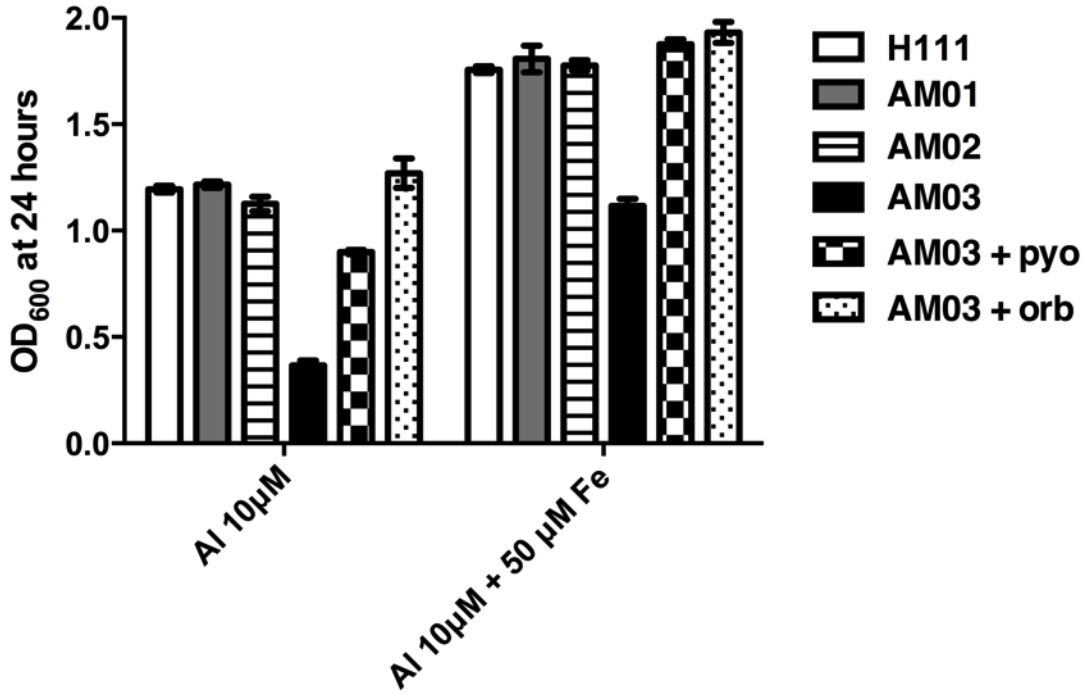

Figure 33B

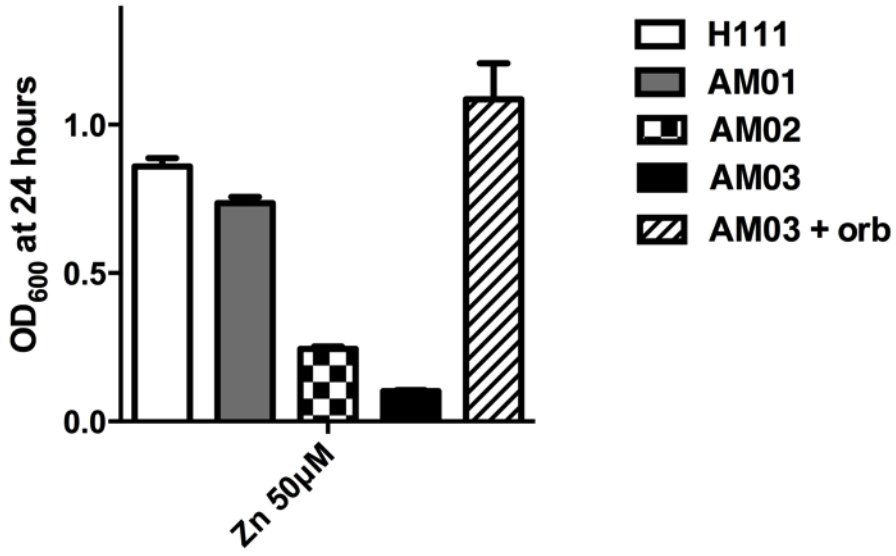

Figure 33.C

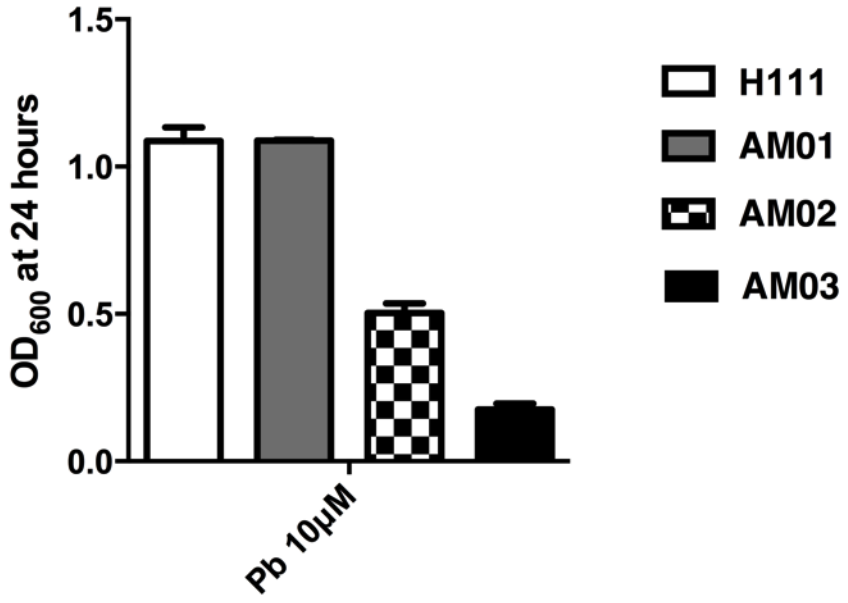

Figure 33.D

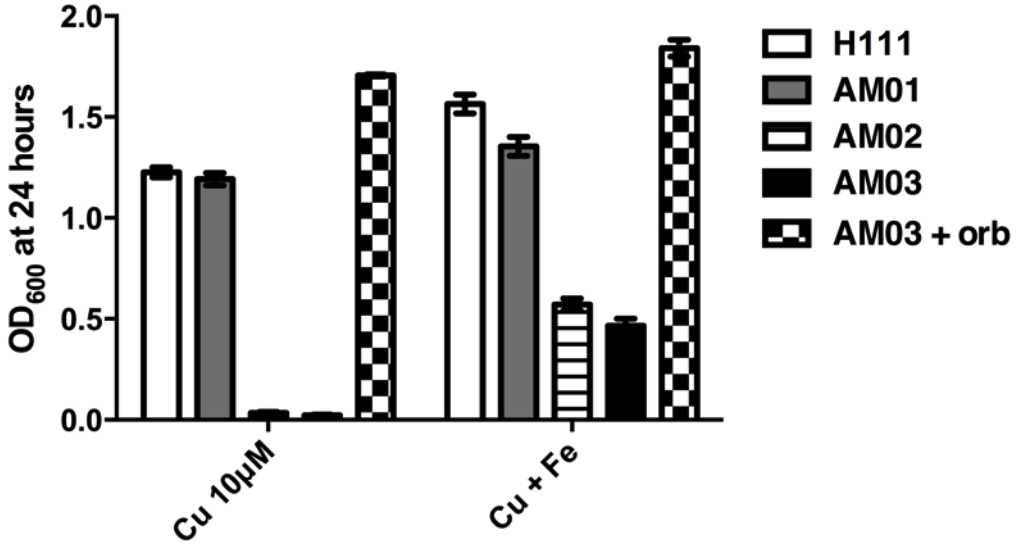

Figure 33: Growth of *B. cenocepacia* H111 and siderophore mutants in iron limited and iron replete media in the presence of (A) 10 µM of aluminium (B) 50 µM of zinc (C) 10 µM of lead and (D) 10 µM of copper. The cultures were incubated for 24 hours and the OD_{600} was measured. The experiment was also carried out in medium supplemented with pyochelin or ornibactin. The experiments with zinc and lead were carried out only in iron-limited medium. Data denotes the mean ± S.E of three independent experiments

6.2.2 The Toxic effect of metals can be relieved by the exogenous addition of ornibactin in *Burkholderia* strains lacking siderophores

While screening for siderophore production by different *Burkholderia* isolates, we observed that five strains did not exhibit any siderophore production on CAS plates. To test whether ornibactin provided protection against toxic metals in these strains, they were grown in medium supplemented with ornibactin extracted from culture supernatants of B*. cenocepacia* H111. Consistent with the protective role of siderophores in H111, the addition of ornibactin alleviated the toxic effect imposed by metals on these strains. While aluminium and lead appeared to be toxic for *B. andropogonis* the addition of 10 µM ornibactin reverted this toxic effect (Figure 34A). Ornibactin provided protection against toxic concentrations of aluminium, lead and copper in *B. tuberum* and *B. sacchari* (Figure 34B and 34C). However, the addition of ornibactin inhibited the growth of the siderophore non-producer *B. mimosarum*, suggesting that this strain lacks the ornibactin receptor and thus iron complexed by ornibactin was not available to the bacterium, resulting in growth inhibition due to iron limitation (Figure 34D). This growth inhibition probably masked the protective effect of ornibactin in this strain.

Figure 34A

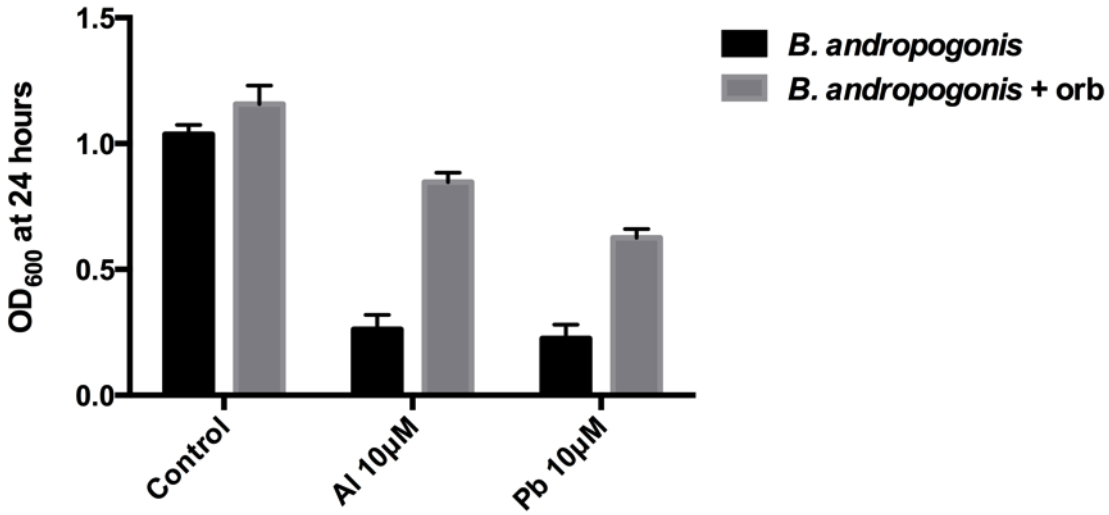

Figure 34B

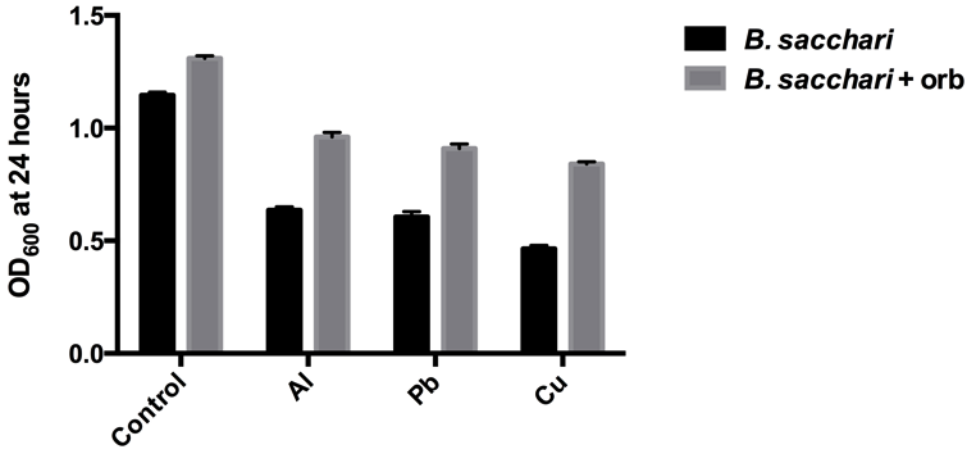

Figure 34C

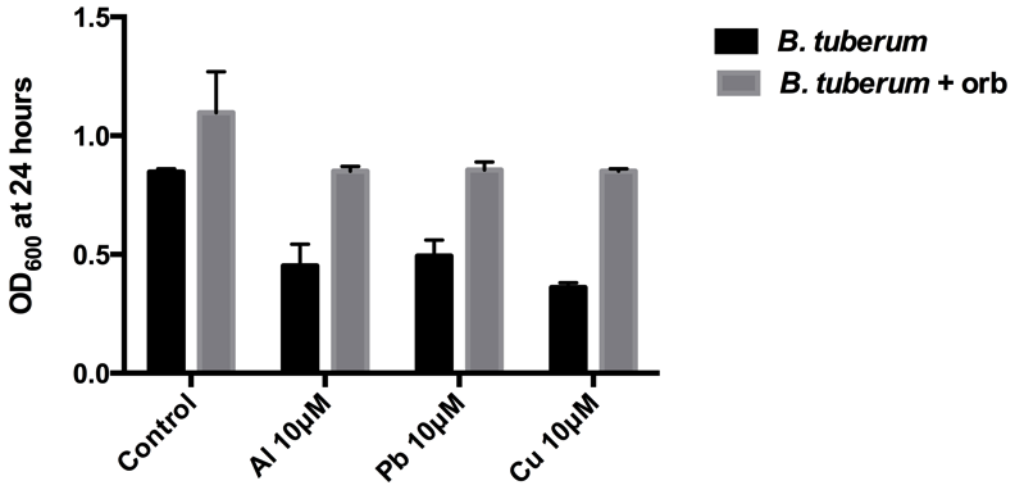

Figure 34D

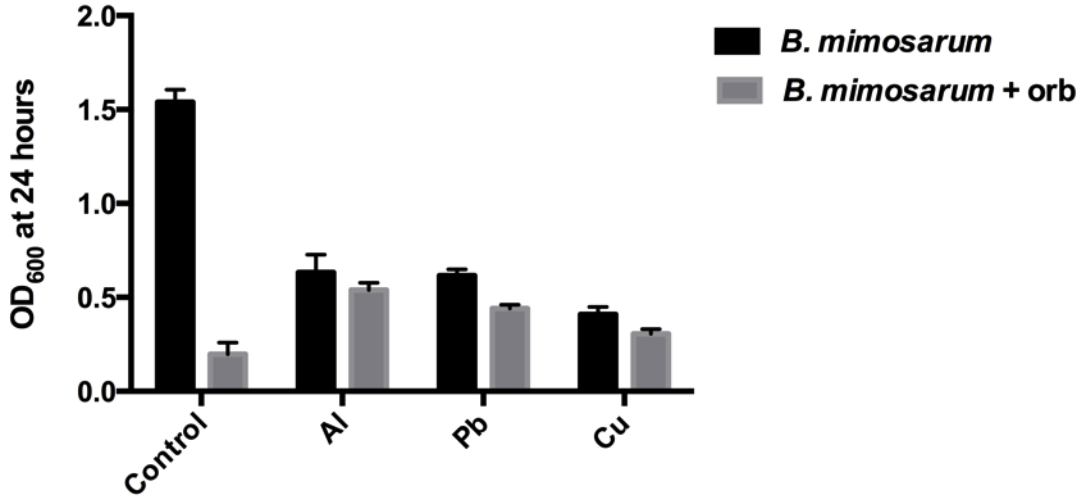

Figure 34: Growth of (A) *B. andropogonis* (B) *B. sacchari* (C) *B. tuberum* and (D) *B. mimosarum* in iron-limited media in the presence of 10 µM aluminium, lead or copper. Growth of *B. andropogonis* in the presence of copper did not yield consistent results and has therefore not been included in the result. Growth assay was also carried out by supplementing the medium with 10 µl of ornibactin extracted from H111 cultures. Data represent mean ± S.E of three independent experiments.

6.2.3 Copper and aluminium induce siderophore production

We hypothesised that if pyochelin or ornibactin are involved in sequestering metals to protect the bacteria, metals other than iron may also regulate their expression. To test this, promoter fusions of the ornibactin biosynthesis operon promoter as well as the pyochelin biosynthesis and transport promoter to *lacZ* were constructed and β-galactosidase activities were measured in the presence of various metals. Since iron is known to induce the expression of pyochelin and ornibactin, the experiment was performed in iron limited and replete conditions. This allowed differentiation of the effects of iron limitation from those caused by the presence of other metals on the regulation of siderophore expression. Since pyochelin production appeared to be induced under iron replete conditions, even in the absence of other metals, these data were not taken into account. However, ornibactin expression was found to be significantly upregulated in the presence of copper in iron replete as well as iron limited conditions and to a lesser extent in the presence of aluminium (Figure 35). This suggests that these metals are able to induce ornibactin expression. However, the exact regulatory mechanism remains to be elucidated.

Figure 35A

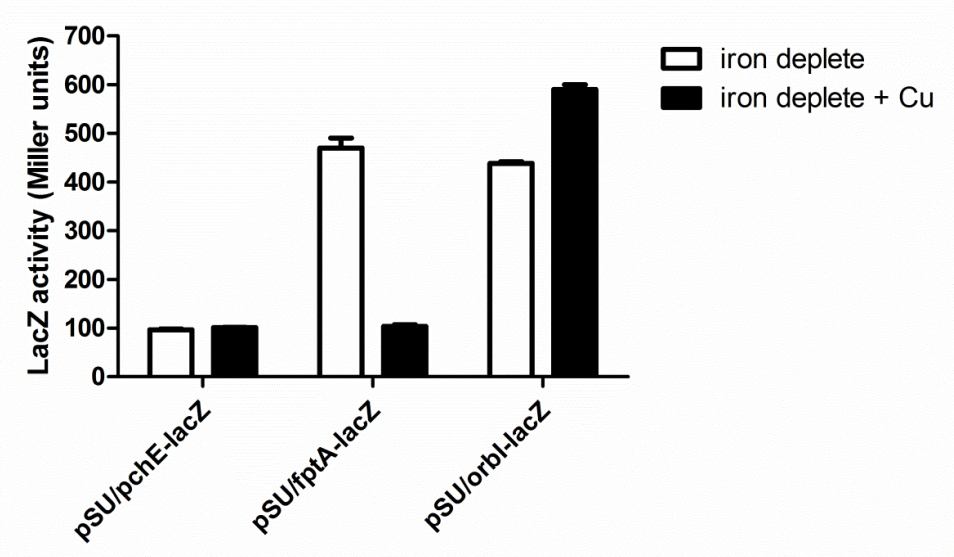

Figure 35B

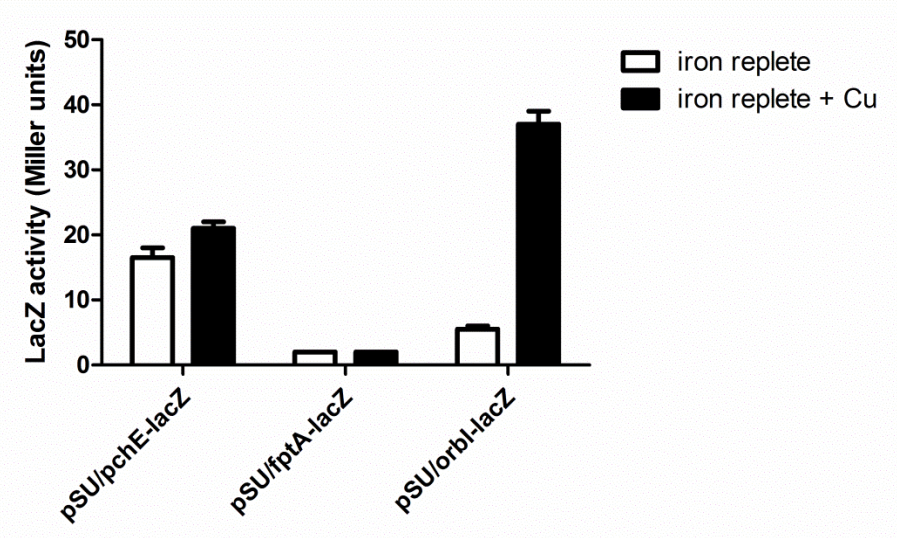

Figure 35C

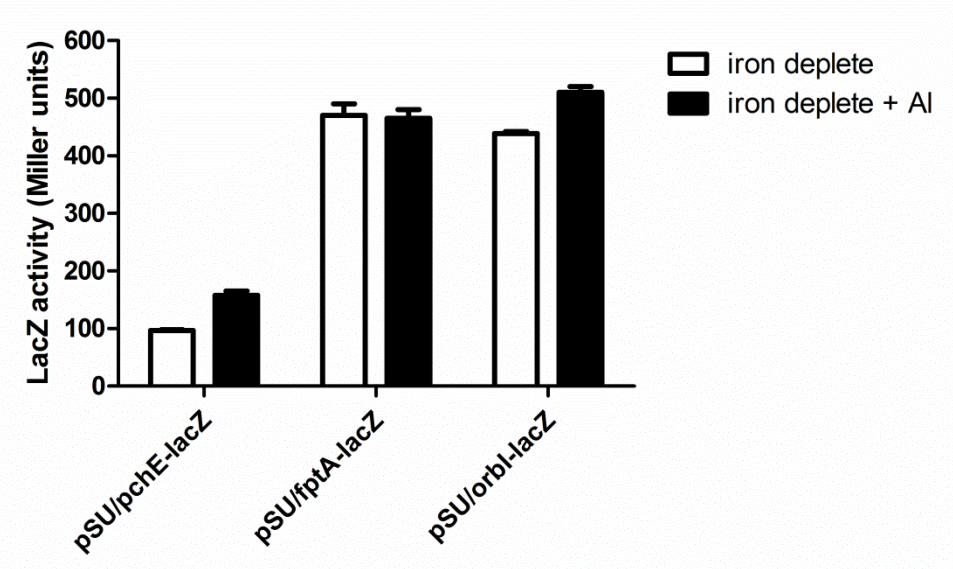

Figure 35D

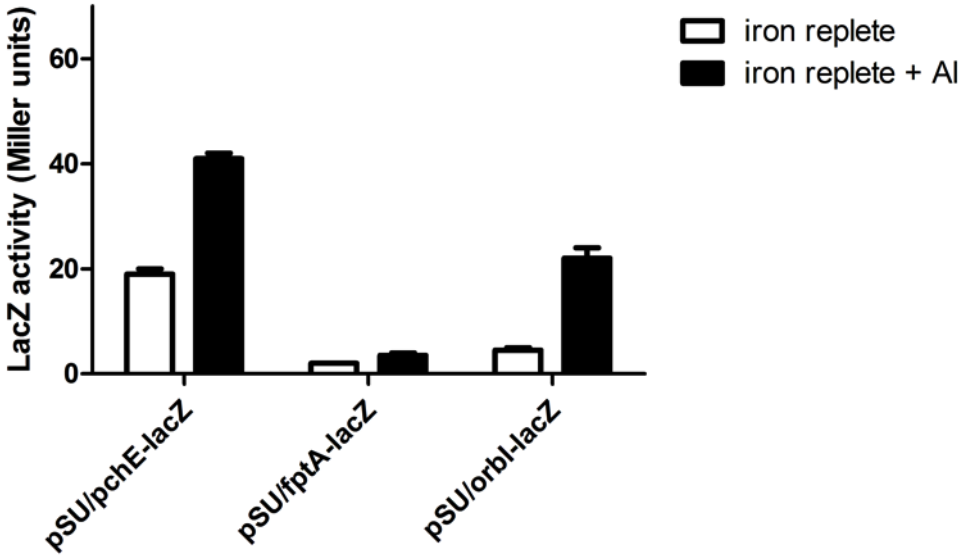

Figure 35: Effect of copper and aluminium in the expression of ornibactin biosynthesis (*orbI-lacZ* fusion) as well as pyochelin biosynthesis (*pchE-lacZ* fusion) and transport (*fptA-lacZ* fusion). β-galactosidase activities were measured in the presence of 10 µM (A) copper in iron deplete or (B) iron replete conditions. The experiment was performed in the presence of 10 µM (C) Al in iron replete and (D) iron replete conditions. Data represent mean ± S.E of three independent experiments.

6.3 Discussion

Like many other bacteria, *Burkholderia* possess different iron transport systems. At least four different siderophores have been isolated from *Burkholderia* strains. The redundant production of siderophores by one organism suggests that they may have alternative and specific roles beyond iron transport. For example, the hydroxamate siderophore ferrioxamine has been shown to inhibit biofilm formation in *Mycobacterium* species (Ishida *et al.*, 2011) and a siderophore from the marine bacterium *Aureobasidium pullulans* was shown to inhibit growth of other competitor bacteria (Chi *et al.*, 2012). Furthermore, it was reported that catecholate siderophores protect *E. coli* cells from the toxic effects of reactive oxygen species from pyochelin toxicity (Adler *et al.*, 2012) and therefore is involved in *E. coli* colony development (Adler *et al.*, 2014). In line with this, alternative biological functions were shown for the siderophores pyoverdin and pyochelin produced by *P. aeruginosa.* These siderophores sequester metals other than iron and thus contribute to metal resistance (Braud *et al.*, 2010). Moreover, the pyoverdin genes

were reported to be upregulated in *P. aeruginosa* strains exposed to copper (Teitzel *et al.*, 2006).

While studying the importance of siderophores in *Burkholderia*, we observed that siderophores are produced by the majority of *Burkholderia* strains. However, their presence did not seem to be essential for iron acquisition in these strains as they were equipped with alternative iron transport systems. Considering the prevalence of *Burkholderia* in soil and the rhizosphere, an alternative role of siderophores in combating metal toxicity in contaminated environments is plausible. The ability of ornibactin producing *Burkholderia* strains to grow in the presence of aluminium, zinc, lead or copper as compared to a mutant strain indicates that this siderophore can protect the cells against these metals. In contrast to in *P. aeruginosa*, pyochelin did not exhibit a significant protective effect, except against aluminium. This protective effect suggests that either siderophores prevent the entry of these metals into the cell or that more efficient iron assimilation in these strains leads to increased growth. Although ornibactin exhibited a positive effect on growth due to improved iron acquisition, the additional positive effects in the presence of aluminium and copper could be attributed to the ability of the siderophore to counteract metal toxicity.

It has been postulated that metals diffuse across the outer membrane through porins in Gram-negative bacteria (Nikaido and Vaara, 1985). However, siderophore metal complexes are too large to pass through porins, which have an exclusion limit of approximately 600 Da. It is therefore possible that the siderophores produced by *Burkholderia* sequester the toxic metals in the medium and thus prevent their entry into the cell. A similar protective mechanism has been postulated for *Streptomyces tendae*, which produces hydroxamate siderophores that bind cadmium and by that protect the cells from the toxic effect of this metal (Dimkpa *et al.*, 2009). The production of pyochelin and pyoverdin by *P. aeruginosa* has also been proposed to protect the bacterium from toxic metals (Schalk *et al.*, 2011). The protective mechanism of a siderophore has been demonstrated in uropathogenic *E. coli*, where the siderophore yersiniabactin enables the organism to resist copper toxicity by

binding host derived Cu (II) and preventing its reduction to the more toxic Cu (I) (Chaturvedi *et al.*, 2012).

In line with the above hypothesis, we observed that ornibactin was upregulated in the presence of copper and aluminium in the medium. It is well known that siderophore biosynthesis in bacteria is tightly regulated by the availability of iron in the environment. However, siderophore production as a means to detoxify metals may require that metals other than iron in the extracellular environment also stimulate its production. Indeed, it has been shown that pyoverdin synthesis is induced in the presence of various metals such as copper, nickel and chromium (Braud *et al.*, 2010). However no such induction in pyochelin production was observed in the presence of toxic metals. These observations were based on our study involving a single concentration of the metals. It will be necessary to carry out these experiments in a range of concentrations to analyse the role of these metals in the regulation of ornibactin and pyochelin in better detail.

The molecular mechanism underlying the activation of ornibactin is not known and it is also unclear how other metals regulate ornibactin production. Binding of other metals by the siderophore will probably reduce their concentration thus leading to an activation of siderophore synthesis. It has been reported that *P. aeruginosa* exposed to copper activated pyoverdin biosynthesis and down-regulated the production of the outer membrane pyoverdin transporter (Teitzel *et al.*, 2006). This suggests that the pyoverdin biosynthesis is activated to sequester the toxic metals in the extracellular space and the subsequent down-regulation of transport genes to prevent the entry of the pyoverdin metal complex into the cell. Since the ornibactin biosynthesis and transport genes are part of the same operon, a difference in the expression of these genes could not be investigated in this study.

Our results and previous reports clearly suggest that siderophores may have functions other than iron transport. The protective effect provided by ornibactin and pyochelin as well as the induction of ornibactin biosynthesis by toxic metals points to a potential role of these siderophores in metal homeostasis. Considering the high prevalence of *Burkholderia* strains in soil and the plant rhizosphere, which often

contains high levels of heavy metals, such a defensive mechanism may be highly beneficial for the bacterium.

6.4 Material and methods

6.4.1 Bacterial strains and growth media

Strains and plasmids used in this study are listed in table S1. Wild type B. cenocepacia H111 (Gotschlich *et al.*, 2001) and three mutants AM01, AM02 and AM03 were the main strains used in this study. Construction of these mutants was described previously (Mathew *et al.*, 2014). All growth experiments were performed in iron limited succinate medium (Meyer and Abdallah, 1978) as well as iron limited medium supplemented with 50 µM ferric iron. $AgNO_3$ (Sigma), $AlSO_4$ (Sigma), $CoCl_2.6H_2O$ (Fluka), $CdCl_2$ (Sigma), $CuCl_2.2H_2O$ (Sigma), $FeCl_3$ (Aldrich), $PbCl_2$ (Fluka) and $ZnCl_2$ (Fluka) were the metals used in this study. The metal stocks were made in 0.5 N HCl, 0.5 N HNO_3 or 0.5 N $H2SO_4$ to a final concentration of 10mM or 50mM.

6.4.2.Growth assay

To test the protective effect of siderophores against different metals, wild type and mutant strains of H111 were grown in iron limited as well as iron replete medium for 24 hours in the presence of 10 µM or 50 µM metals and absorbance was monitored at 600 nm. A culture without metals was used as a reference for each strain. Ornibactin and pyochelin crude extracts obtained from H111 culture was added to the growth medium to confirm the protective role of these siderophores.

Similar growth assays were also performed using several *Burkholderia* isolates in iron-limited conditions in the presence of 10 µM metal.

6.4.3.Construction of transcriptional *lacZ* fusions and beta galactosidase assays

Transcriptional fusions of promoters driving expression of the ornibactin biosynthesis gene (*orbI*) as well as pyochelin biosynthesis (*pchE*) and transport (*fptA*) genes were constructed as follows. The three promoter regions were amplified using primers listed in table S2 and digested with XhoI and HindIII to be inserted into the reporter vector pSU11 (Malott *et al.*, 2009). The resulting plasmids pSU/pchE-lacZ,

pSU/fptA-lacZ and pSU/orbI-lacZ were transferred to *B. cenocepacia* H111 by triparental mating.

Beta galactosidase activity of the reporter was measured as previously described (Stachel *et al.*, 1985) with some modifications. Briefly, cells grown in the presence of different metals were normalised by cell growth, harvested and resuspended in 1ml Z buffer. Cells were lysed using 25 µl of CHCl3 and 25 µl of 0.05 % SDS followed by incubation at 30 °C for 10 minutes. The reaction was initiated by adding 200 µl ONPG (4 mg/ml) and incubated at 30 °C. After terminating the reaction using 1 M Na_2CO3, cell debris was removed and absorbance was measured a 420 nm. Beta gal activity was recorded as Miller units using the formula (1000*OD420)/(time [min]*V [ml]*OD600).

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Chapter 7. Discussion and outlook

7. 1 Discussion

Members of the genus *Burkholderia* are known to acquire iron by means of siderophores. The importance of different siderophore systems for growth of *Burkholderia* sp. under iron limiting conditions has been well studied. *Burkholderia* species have also been reported to utilise heme, ferritin and lactoferrins as important iron sources during infection and are therefore considered important virulence factors. In this work, we show that all investigated *Burkholderia* species possess an alternative, high affinity iron transport system, FtrABCD, which is highly conserved across *Burkholderia* species. While we could demonstrate that the Ftr system efficiently transports both ferric iron and ferrous iron, the exact mechanism by which the insoluble ferric iron passes through the outer membrane remains to be elucidated. The role of organic acids such as gluconic acid in chelating and transporting ferric iron has also been investigated in this study. Our preliminary observations suggest that *Burkholderia* species secrete compounds which are capable of binding and solubilising iron. However, the chemical nature of these substances has not been identified and their role in transporting iron through the Ftr pathway needs to be verified in future experiments.

Burkholderia species are known to inhabit a wide range of niches ranging from soil and plant rhizosphere to lungs of CF patients. Iron is also known to exist in different forms in these diverse habitats and iron availability is probably an important parameter for the growth of *Burkholderia* strains in these niches. Hence, it is not surprising that they have evolved to utilize multiple iron transport systems that enable them to efficiently assimilate iron under different conditions. In agreement with previous work, my investigations showed that the siderophore-based iron uptake systems are particularly important for virulence of *Burkholderia* strains (Visser et al., 2004; Uehlinger et al., 2009; Mathew et al., 2014). The finding that a harmless environmental *Burkholderia* strain, *B. sacchari,* became pathogenic upon heterologous expression of ornibactin confirms the importance of this siderophore in virulence. In addition, this finding also suggests that a single virulence determinant

is sufficient to render a *Burkholderia* strain belonging to the plant-associated beneficial clade pathogenic, at least in an invertebrate infection model.

Although several *Burkholderia* species that are associated with plants or soil have been identified and characterised, iron transport mechanisms in this environmental *Burkholderia* clade of have not been investigated. In the second part of my study, I focused on the ecophysiological role of siderophore systems and the Ftr system. To this end, I investigated the importance of siderophores and the Ftr system for growth of *Burkholderia* in soil as well as for the symbiosis of nodulating *Burkholderia* strains with legumes. While none of these systems played a significant role in nodulation of legumes, the Ftr system was found to be important for survival and persistence in soil. Subsequently, I determined the role of siderophores in heavy metal resistance in *Burkholderia*. These experiments showed that production of ornibactin had a protective effect against toxic metals, suggesting an alternative role for the siderophore other than iron transport and virulence.

Overall, this study characterizes a novel iron transport system and provides insight into the specific roles of siderophores and the Ftr transport system in diverse environmental niches.

7.2 Outlook

Ftr system: A unique transport system different from Ftr systems of *Bordetella* and *Brucella* species?

In the framework of this thesis I have shown that the Ftr_{Bcc}ABCD system of our model strain *B. cenocepacia* H111 efficiently transports ferric iron. This is in stark contrast to the orthologous Ftr systems of *B. pertussis* and *B. abortus*, whose components share 50 % identity to the Ftr_{Bcc} system (Brickman and Armstrong, 2012; Elhassanny *et al*., 2013). Based on its importance for the growth of these pathogens at acidic pH and its expression in response to iron deprivation at low pH, the Ftr system has been proposed to be a ferrous iron transport system in *B. pertussis* and *B. abortus*. Although the Ftr_{Bcc}ABCD system can also transport ferrous iron, it has a higher affinity for ferric iron,suggesting that there are major differences in the substrate specificity of the different Ftr systems (Mathew *et al*., 2014). Moreover, it has been shown that the Ftr system is indispensable for the virulence of *Brucella*

strain unlike the Ftr_{Bcc} system, which was found not to contribute to Bcc virulence (Elhassanny *et al*., 2013). Interestingly, siderophores were shown to be irrelevant in establishing infection by *B. abortus* in a previous study (Bellaire *et al*., 1999; Gonzalez Carrero *et al*., 2002). It has been proposed that *B. abortus*, being an intracellular pathogen, utilizes the Ftr system to acquire iron in its ferrous form from the host's intracellular compartments, thus explaining the critical role of the Ftr system in virulence. In contrast, *B. cenocepacia* colonizes the lungs of CF patients where iron is predominantly sequestered by transferrin, lactoferrin, ferritin and haemin. Siderophores and proteases may therefore be crucial for acquiring iron from such extracellular sources. These observations on homologous Ftr systems, as well as our findings, indicate that there is considerable difference in the role and functioning of the Ftr_{Bcc} system compared to homologous Ftr systems.

Based on our results and orthologous proteins in yeast as well as bacterial iron transport systems, we hypothesised that Ftr_{BCC} system functions as follows:

Ferrous or ferric iron is the initial substrate and binds to the p19 protein ($Ftr_{Bcc}A$), which is involved in high affinity iron transport. The ferric iron is then reduced and transferred to the Ftr1 permease protein ($Ftr_{Bcc}C$), which subsequently is transported across the outer cell membrane. The cupredoxin domain protein $Ftr_{Bcc}B$ and the iron sulfur cluster of ferredoxin ($Ftr_{Bcc}D$) are thought to be involved in electron transfer and thus iron reduction during transport (Figure 36).

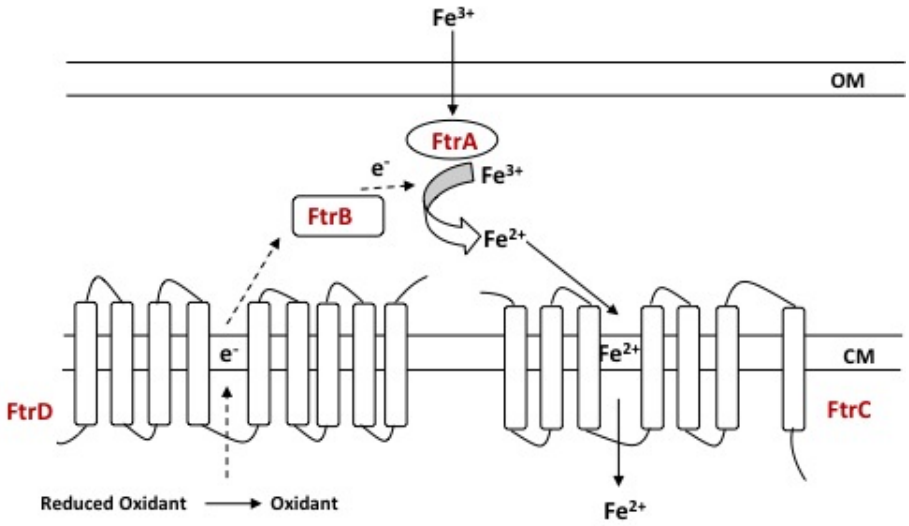

Figure 36: Hypothetical model for the transport of iron by Ftr_{Bcc} system in *Burkholderia* species

The individual roles of the different Ftr proteins in this iron transport pathway need to be verified by biochemical analyses. However, I have demonstrated that Ftr can utilise both ferric and ferrous iron as its initial substrate. Additionally, it was also shown that the Ftr system possesses reductase activity, although the reductase component could not be identified. Individual Ftr proteins have to be purified and characterised to evaluate their specific roles in better detail.

Ornibactin, a virulence determinant in *Burkholderia* strains?

One of the most interesting findings of this study was the gain of pathogenic potential by an environmental *Burkholderia* strain upon introduction of the ornibactin biosynthesis gene cluster. While the transgenic soil isolate *B. sacchari* was found to be virulent in a *G. mellonella* infection model, heterologous expression of ornibactin in a nodulating *B. tuberum* did not increase virulence of the strain. Although both strains were tested for some potential virulence factors such as protease production and biofilm formation, a detailed bioinformatic analysis of their genomes will be required to evaluate the different behaviors of the transgenic strains. It remains to be elucidated whether a specific genetic strain background is required that, in concert with the production of ornibactin, is sufficient to render an environmental, avirulent *Burkholderia* strain pathogenic. Further experiments with transgenic environmental *Burkholderia* isolates need to be performed in different infection hosts to verify the role of ornibactin as a key virulence factor. Moreover, investigations on the expression of ornibactin in *B. sacchari* inside *G. mellonella* larvae could confirm that the pathogenicity acquired by the strain is indeed due to ornibactin production and not due to unknown regulatory effects.

What is the iron transport strategy of *Burkholderia* bacteroids in the legume nodules?

Our study on the importance of siderophores and Ftr system in nodule infection by *B. tuberum* revealed that neither siderophores nor the Ftr system is required for nodulation and symbiosis of the bacterium with legumes. However, the major

strategy of iron acquisition by the bacteroids in the nodule has not been identified. Investigation on the importance of other iron transport strategies such as the iron citrate transport system in nodulation and symbiosis would be a highly interesting field for further investigations. An alternative approach, namely transcriptomics of cells isolated from nodules is currently ongoing in our laboratory and may provide information on how these cells assimilate iron.

Is the Ftr system crucial for growth of *Burkholderia* isolates in soil?

Our preliminary results show that the Ftr system is important for growth of *Burkholderia* strains in soil microcosms. This finding is based on results obtained with the *B. cenocepacia* H111 strain. Investigations of growth and persistence of soil and plant-associated *Burkholderia* isolates with only siderophore- or Ftr-based iron uptake systems in soil microcosms would allow us to confirm the distinct role of the Ftr system for growth in soil.

Do metals other than iron regulate siderophore production?

The study of possible alternative roles of siderophores in metal homeostasis revealed that they exhibit a protective effect against toxic concentrations of heavy metal. In line with this finding, we observed that a high copper concentration up-regulates the expression of the siderophore ornibactin, suggesting that ornibactin production was activated to sequester the toxic metal in the medium. Whether this regulation is through the Fur system or another, as yet unidentified regulatory network, needs to be elucidated.

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Chapter 8. Material and methods

8.1 Bacterial strains, plasmids and oligonucleotides

All strains, plasmids and oligonucleotides used in this study are listed in table 6.1, 6.2 and 6.3 respectively

Table 7: Bacterial strains used in this study

Strain name	Genotype/characteristic	Reference
***Escherichia coli* strains**		
Top 10		Invitrogen
CC118 λpir	Δ(*ara, leu*)7697 *araD*139 *ΔlacX*74 *galE galK pho*A20 thi-1 *rps*E *rpoB(*RfR) *argE*(am) *recA1* λpir+	(Herrero et al., 1990)
GR536	K12 (W3110) derivative *ΔfecABCDE*::kan *ΔzupT*::cat *ΔmntH ΔfeoABC* Δ*entC*	(Grass et al., 2005)
S17	thi *recA* pro hsdR- hsdM+ RP4-2-Tc::Mu-Km::Tn7	(Simon et al., 1983)
Replicator v2.0	F´ *mcrA* Δ(mrr-hsdRMS-mcrBC) endA1 *recA*1 ϕ80d*lacZ*ΔM15 *ΔlacX*74 *araD*139 Δ(ara,leu)7697 *galU galK rpsL* (StrR) *nupG* (attL araC-PBAD- trfA250 bla attR) λ–	Lucigen
H1717	*araD139 ΔlacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF*:: λplacMu	(Hantke, 1987)
OP50	Food source for *C. elegans*; Uracil auxotroph	(Brenner, 1974)
***Burkholderia cenocepacia* strains**		
H111	Clinical isolate, Wild type	(Gotschlich et al., 2001)
BccAM01	H111 *ΔpchAB*	(Mathew et al., 2014)

BccAM02	H111 *ΔorbJ*	(Mathew et al., 2014)
BccAM03	H111 *ΔpchAB ΔorbJ*	(Mathew et al., 2014)
BccAM04	H111 *ftr$_{Bcc}$C*:: pSC200, Tp^R	(Mathew et al., 2014)
BccAM05	H111 *ΔpchAB ΔorbJ ftr*$_{Bcc}$C ::pSC200	(Mathew et al., 2014)
BccAM06	BccAM03 complemented with pAUM1	(Mathew et al., 2014)
BccAM07	BccAM04 complemented with pAUM2	(Mathew et al., 2014)
BccAM08	H111 *ftr$_{Bcc}$A*:: pSC200, Tp^R	This study
BccAM09	H111 *ftr$_{Bcc}$B*:: pSC200, Tp^R	This study
BccAM10	H111 *ftr$_{Bcc}$D*:: pSC200, Tp^R	This study
B. tuberum	Root nodule isolate from tropical legume	(Vandamme et al., 2002)
AM11	B. tuberum *ftr$_{Bcc}$C*:: pSC200, Tp^R	This study
B. sacchari	Soil isolate from sugarcane plantation	(Bramer et al., 2001)
AM12	*B. sacchari ftr$_{Bcc}$C*:: pSC200, Tp^R	This study
AM13 (*B. tuberum* orb)	*B. tuberum* carrying ornibactin locus	This study
AM14 (*B. sacchari* orb)	*B. sacchari* carrying ornibactin locus	This study

Table 8: Plasmids used in this study

Plasmid	**Genotype/ description**	**Reference**
pBBR1MCS-5	Broad host range vector, Gm^R	(Kovach et al., 1995)
pAUM1	pBBR1MCS-5 with orbJ	(Mathew et al., 2014)
pAUM2	pBBR1MCS-5 with f*tr$_{Bcc}$ABCD*	(Mathew et al., 2014)
pAUM3	pBBR1MCS-5 with f*tr$_{Bcc}$BCD*	(Mathew et al., 2014)

pAUM4	pBBR1MCS-5 with f*tr*$_{Bcc}$*ACD*	(Mathew et al., 2014)
pAUM5	pBBR1MCS-5 with f*tr*$_{Bcc}$*ABD*	(Mathew et al., 2014)
pAUM6	pBBR1MCS-5 with f*tr*$_{Bcc}$*ABC*	(Mathew et al., 2014)
pDONR221	Gateway adapted donor vector; attP1 and attP2, ccdB, pUC origin, Cm^R	Invitrogen
pAUC40	Gateway compatible suicide vector: R6K origin, $Strep^R$ and Cm^R, sacB, ccdB, attR1 and attR2	(Carlier et al., 2009)
pSC200	prha*B* rhamnose inducible promoter, *rhaR, rhaS* and dhfr cassette, Tp^R	(Ortega et al., 2007)
pAUM7	pSC200 containing 300 bp *ftrBccC* fragment	(Mathew et al., 2014)
pRK600	RK2-mob^+ RK2-tra^+, ori ColE1	(Kessler et al., 1992)
pRK2013	RK2 derivative, mob^+ tra^+ ori ColE1	(Figurski & Helinski, 1979)
pSU11	Promoter probe vector, Gm^R	(O'Grady et al., 2009)
pFTCAPL-GW	ori*Tn*7 left capture vector; Gateway destination vector, mini-F copy control replicon, Km^R Cm^R	(Kvitko et al., 2013)
pUCTCAPR2-Tp^R	ori*Tn*7 right capture vector; Gateway destination vector, pUC replicon, FRT-ZeoR-FRT cassette is swappable by XbaI digest; Tp^R Gm^R Cm^R	This study
pBBRK2013	pBBR conjugation helper plasmid; Gm^R Km^R	(Kvitko et al., 2013)
pSU11/ppchE	Promoter probe vector pSU11 containing the pchE (pyochelin biosynthesis gene) promoter region, Gm^R	Silja Inhülsen (unpublished)
pSU11/pfptA	pSU11 containing the *fptA* (encodes ferric-pyochelin receptor) promoter region, Gm^R	This study

pSU11/porbI	pSU11 containing the *orbI* (encodes NRPS involved in ornibactin synthesis) promoter region, Gm^R	This study
pOrnibactin-Tn7	Mini F replicon carrying the ornibactin locus, Km^R, Tp^R	This study
pDONR-orbL	Directional gateway entry vector carrying left flanking region of ornibactin locus, Km^R	This study
pDONR-orbR	Directional gateway entry vector carrying right flanking region of ornibactin locus, Km^R	This study
pFTCAPL-orbL	LR recombinant of pFTCAPL-GW and left ornibactin flanking fragment, Km^R	This study
pUCTCAPR-orbR	LR recombinant of pUCTCAPR2-Tp^R and right ornibactin flanking fragment, Tp^R, Gm^R	This study
pTNS3	Tn7 transposase helper plasmid, site-specific TnsD- pathway; pir-dependent replicon, Amp^R	(Choi et al., 2008)
pBluescript II KS	High-copy-number cloning vector; Amp^R	Stratagene
pBluescript/p*ftrA*	pBluescript containing putative *ftr* locus promoter region, Amp^R	This study

Table 9: Oligonuceotides used in this study

Primer name	**Primer sequence**
Primers used for cloning ornibactin locus into *Burkholderia* strains	
orb_L_attB1_fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGAGTCAGG CCGTCGATAC
orb_L_attB2_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTAGATCGGG AACGACAGGAT
orb_R_attB1_fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTCATCAAGGA GGTCGTGCAT
orb_R_attB2_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTCAATG CTTCCTGCAAT
att_fwd	CCATCAAACCACGTCAAA
Kan_Dn_rev	CGAAATGACCGACCAAGCGA
att_rev	TACGATACACTTCCGCTCA
Kan_Up_fwd	ACGTGTTCCGCTTCCTTTAGC
CAPL_orbL_fwd	ACGACATCAGTTTGCTCCT
Primers used to exchange the resistance cassette in CAPR-plasmid for ornibactin introduction	
trp_frt_fwd	GGGGTCTAGAGAATAGGAACTTCGGAATAGGAACTTCCAGTT

	GACATAAGCCTGTTCGG
trp_frt_rev	GGGGTCTAGAAAGTATAGGAACTTCTTAGGCCACACGTTCAA GTGC
Primers used to make Ftr system mutants	
ftrA_mut_fwd	GCTCGAGTACGATTTCCC
ftrA_mut_rev	GGACACTCCTTGTTATTTG
ftrB_mut_fwd	TTTTCGACGATTTCCACC
ftrB_mut_rev	CATTCTGGGTTCTCTACG
ftrC_mut_fwd	GATCCTCGATGATTCGGG
ftrC_mut_rev	CCATCGATACCTCCTGAC
ftrD_mut_fwd	GCATGGTGGTTGATGTTC
ftrD_mut_rev	GACGATCCACTGGATACC
Primer pairs used to test the presence of *ftr* locus	
ftrA_fwd	TGTTGGGTTCTTCGTTCAT
ftrA_rev	TTCGTCAGCTTGTACGTG
ftrB_fwd	TGAAAATTCCCCAGAAAATCG
ftrB_rev	CTGCACGCTCTCGAATTC
ftrC_fwd	TGGGTCAGATCTTGTTCAT
ftrC_rev	CCGTACAGGAAGATCACC
ftrD_fwd	TGAGCGTTGTCGCCGCCG
ftrD_rev	CGCTCACCATCTGTCCGT
Primers used to make Ftr promoter fusion constructs	
ftrA_Xho1_fwd	GGGGCTCGAGAATCTTTCCAAGCGTCAC
ftrA_Hind111_rev	GGGGAAGCTTATGAACGAAGAACCCAAC
ftrC_Xho1_fwd	GGGGCTCGAGGAATTCGAGAGCGTGCAG
ftrC_Hind111_rev	GGGGAAGCTTACGATGAACAAGATCTGACC
Primers used for Fur titration assay	
pfrt_BamH1_fwd	GGGGGGATCCTCTTGACCGCATGGATGT
pfrt_EcoRV_rev	GGGGGATATCGCAACTCGCGAAGGAAAT
Primers used to clone *ftr* locus into *E. coli*	
ftrloc_fwd_sma1	GGGGCCCGGGATCTTTCCAAGCGTCACT
ftrloc_rev_hind	GGGGAAGCTTGAGAGGTGTTGGGCAGTA
Primers used to test the insertion of the ornibactin locus into *Burkholderia* strains	
orbS_fwd	CGTTCCTCGGCAGTTATC
orbS_rev	TGATCGGAAATCGCTGGG

orbG_fwd	ATGACCCTGCTTTCGTTG
orbG_rev	TTCCTGCATGTCGTCGTC
orbF_fwd	ACCTGCTCGATACCTGTC
orbF_rev	GATGAGATCCGGCTTGAC
orbI_fwd	CACATGACGAGTTTCCCG
orbI_rev	GACGCGAAATGCAGCAGA
pvdA_fwd	CGTATTTCGAGGCGTTCT
pvdA_rev	GACGACGAATGGATCACG
orbA_fwd	CGCGTCCTATGTCTACCA
orbA_rev	GGATCGAACAGAACAGCG
orbJ_fwd	GGATCCCGGATGACGAAGGTGCAA
orbJ_rev	CCCGGGGAGGCGGTAGGTATCGAG
Primers for ornibactin promoter fusion constructs and for verifying the construct	
pOrbI_Xho_fwd	GGGGCTCGAGCAGCAGCGCAAGGATCAG
pOrbI_Hind_rev	GGGGAAGCTTTCGTTCTGGAAAGGCACC
pSC_fwd	GTC ATA CTG GCC TCC TGA TGT CGT C
lacZ-rev	TGCTGCAAGGCGATTAAG
Primers for qPCR to check for *ftr* as well as control (ornibactin biosynthesis gene) expression under different conditions	
orb_qpcr_fwd	CGGTCTTCATCGACAAAC
orb_qpcr_rev	CGCTCGGTGATGTGATAC
ftrA_F_qpcr	CGTACAAGCTGACGAAGCAG
ftrA_R_qpcr	ATCGTACTCGAGCGTGATGG
ftrC_F_qpcr	GCGCAGGACTATTTCCAGAC
ftrD_R_qpcr	GAGGCCGTACAGGAAGATCA
frt_seq_fwd	GTACAAAGTGGTGATGGG
frt_seq_rev	TAGGACAAATCCGCCGCT

Primers used to check the correct insertion of ornibactin locus upstream of *glmS* gene in *Burkholderia* strains	
cag_fwd (for *B. sacchari*)	GTCATACTGGCCTCCTGATGTCGTC
glmS_fwd (for *B. tuberum*)	CAGCGCAACCGACAGAAA
TN7_L	ATTAGCTTACGACGCTACACCC
Primers used for qPCR to check the expression of *ftr* genes as well as house keeping genes in *B. tuberum*	
tuberum_recA_fwd	GTCGAAATCTACGGACCGGA
tuberum_recA_rev	ATTTCGAGCGCCTGTTCTCC
rpoD_fwd	GATGCAACCCAGACCGAAGA
rpoD_rev	TCCTTGCCGAGCTTGATCAG

8.2 Media and growth conditions

All the solutions and media were prepared with distilled water (dH2O), unless otherwise stated and sterilized for 20 min at 121 °C and 1.2 bar (OmegaTM 121/134, PrestigeTM Medical, Blackburn, GB). For solid media, 1.5 % (w/v) agar was added prior to autoclaving. In general, strains were incubated at 37 °C overnight, unless otherwise indicated. The media were from Becton-Dickinson (Sparks, USA) and chemicals from Fluka (Buchs, Switzerland) or Sigma-Aldrich (Buchs, Switzerland). The enzymes and reagents used for molecular biological work were purchased from Microsynth (Switzerland), MBI Fermentas (Glen Burnie, USA), New England Biolabs (Ipswich, USA), Promega (Madison, USA), TAKARA (Osaka, Japan) or Vaudaux-Eppendorf (Hamburg, Germany). For DNA work and preparation of iron-limited media, highly purified and autoclaved Milli Q water was used (Millipore, Billerica, USA). Bacterial cultures were generally stored in 25 % glycerol at -80 °C. Media used in this study are described below.

8.2.1. LB (Luria-Bertani) medium (Bertani, 1951)

Casein hydrolysate (Bacto tryptone, BD)	10 g
Yeast extract (Bacto yeast extract, BD)	5 g
NaCl	4 g
dH_2O	1000 ml

8.2.2. Pseudomonas Isolation Agar (PIA)

PIA*	45 g
Glycerol	20 ml
dH_2O	1000 ml

*Becton, Dickinson and Company, Sparks, USA

8.2.3. SOC medium (Hanahan, 1983)

Bacto™ Tryptone	20 g
Bacto™ yeast extract	5 g
$MgCl_2 \times 6H_2O$	2 g
$MgSO_4 \times 7H_2O$	2.5 g
Glucose	3.6 g
dH_2O	1000 ml

pH 7.0

8.2.4. CAS medium

This medium is a modified version of the original CAS medium (Schwyn *et al*., 1987). Iron was supplied in a complexed form with a dye, chrome-azurol. Upon siderophore production, the iron was dissociated from the dye and the medium turned from blue to orange.

Solution 1

Chrome azurol S	0.06 g

MilliQ	50 ml

Solution 2

HDTMA ($C_{19}H_{24}BrN$)	0.07 g
MilliQ	40 ml

Fe^{3+} solution

$Fecl_3$	1 mM
Hcl	10 mM
MilliQ	100 ml

- Freshly prepared Solution 1 and 2.
- Gently added 10ml of Fe^{3+} solution to solution 1
- Added solution 2 to obtain Chrome S/Fe^{3+}/HDTMA complex, autoclaved and kept at room temperature in the dark.

Mixture solution:

Minimal Media 9 (MM9) Salt Solution Stock

15 g KH_2PO_4, 25 g NaCl, and 50 g NH_4Cl in 500 ml of ddH_2O

20 % Glucose Stock

20 g glucose in 100 ml of ddH_2O

NaOH Stock

25 g of NaOH in 150 ml ddH_2O and pH ~12

Casamino Acid Solution

3 g of Casamino acid in 27 ml of ddH_2O

Filter sterilized

CAS agar Preparation:

- Added 100 ml of MM9 salt solution to 750 ml of ddH_2O
- Dissolved 32.24 g piperazine-N,N'-bis(2- ethanesulfonic acid) PIPES
- Added 15 g Bacto agar

- Autoclaved and cool to 50 °C
- Added 30 ml of sterile Casamino acid solution and 10 ml of sterile 20 % glucose solution to MM9/ PIPES mixture
- Slowly added 100 ml of Blue Dye solution along the glass wall with enough agitation to mix thoroughly

8.2.5 CAS liquid assay solution (Smith, 1998)

Stock solutions

1. 2 mM Chrome azurol S stock solution (0.121 g CAS in 100 ml dH_2O)
2. 1 mM Fe stock solution (1mM $FeCl_3.6H_2O$ in 10 mM HCl)
3. 0.2 M 5-Sulfosalicylic acid in dH_2O

CAS liquid medium preparation

- Dissolved 4.3 g Piperazine in 30 ml water and adjusted the pH to 5.6 by addition of 6.25 ml concentrated HCl.
- Dissolved 0.02 g Hexadecyl trimethyl ammonium bromide (HDTMA) in 50 ml dH2O in a separate flask.
- Added 7.5 ml CAS solution, 1.5 ml Fe solution, the piperazine solution and 2 ml 5-Sulfosalicylic acid while stirring and adjusted the volume to 100 ml.

8.2.6. Iron limited succinate (IFS) medium (Meyer and Abdallah, 1978)

K_2HPO_4	6.0 g
KH_2PO_4	3.0 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4$ x$7H_2O$	0.2 g
Succinic acid	4.0 g
MQ H_2O	1000 ml;
	pH 7.0

8.2.7. Nematode growth Medium (NGM II) (Mahajan-Miklos *et al*., 1999; Tan et al., 1999) for *C. elegans*

NaCl	51 mM
Bactopeptone	0.35 % (w/v)
Agar	1.7 % (w/v)
Additives	(see below)

Additives (sterile, final concentration)

KPO_4 buffer pH 6.0	25 mM
$CaCl_2$	1 mM
$MgSO_4$	1 mM
Uracil	2 µg ml^{-1}
Cholesterol	5 µg ml^{-1}
Nystatin solution	50 µg ml^{-1}

Nystatin solution

Nystatin	10 mg ml^{-1}

In 1:1 mixture of 100 % Ethanol and 7.5 M ammonium acetate

KPO_4 buffer pH 6.0

KH_2PO_4	0.8 M
K_2HPO_4	0.2 M

pH 6.0

The additives were autoclaved or sterile filtered separately and added when the media had cooled to 50 °C.

8.2.8. Inhibitors and additives

Media were supplemented with antibiotics or inhibitors to select the mutants. Heat sensitive additives were filter sterilized (22 µm pore size) and were added to the respective media before inoculation.

Table 10: Concentrations of antibiotics/chelators used

Antibiotic/additives	***E. coli* strains**	***Burkholderia* strains**
Ampicillin	100 µg/ml	-
Chloramphenicol	20 µg/ml	20 µg/ml
Gentamicin	10 µg/ml	20 µg/ml
Kanamycin	50 µg/ml	100 µg/ml
Streptomycin	50 µg/ml	100 µg/ml
Trimethoprim	25 µg/ml	50 µg/ml
2-2' Bipyridine	-	30 µM
Desferoxamine		10 µM

8.3 Molecular methods

All standard molecular methods were performed essentially as described by Sambrook & Russell, 1989

8.3.1 DNA extraction

8.3.1.1 Plasmid DNA

For the extraction of plasmid DNA from *E. coli* and *Burkholderia* spp. strains, either QiaPrep Spin Miniprep Kit from Qiagen (Hilden, Germany) was used or plasmid DNA was extracted by alkaline lysis with SDS by means of minipreparation, adapted from Molecular Cloning, 3rd edition, by Joseph Sambrook and David W. Russell. DNA from agarose gels was extracted using QiaQuick Gel Extraction Kit and PCR products were purified with the QiaQuick PCR Purification Kit (both from Qiagen).

8.3.1.2 Chromosomal DNA

DNA extraction was based on Kate Wilson's Current Protocols in Molecular Biology (Wilson, 2001). Cells from 5 ml overnight culture of *B. cenocepacia* H111 in LB were harvested at 5000 rpm for 10 minutes and resuspended in 567 µl of TE buffer. Then 30 µl of 10 % SDS and 3 µl of proteinase K (20 mg/ml) were added, mixed and

incubated at 42 °C for 1 h. Before adding 80 µl of CTAB/NaCl solution, the salt concentration in the sample was adjusted by adding 100 µl of 5 M NaCl. The sample was incubated for 10 min at 65 °C. To remove the CTAB- protein/polysaccharide complexes, the same volume of chloroform/isoamyl alcohol (24:1) was mixed with the sample and centrifuged for 5 min. The aqueous supernatant was carefully transferred into a fresh tube and again precipitated with the same volume of phenol/chloroform/isoamyl alcohol (25:24:1). After spinning down, the interface was removed with a sterile toothpick and the supernatant was again transferred into a fresh tube, before the DNA was precipitated with 0.6 volumes isopropanol. The white DNA precipitate that formed after shaking the tube was removed with a pipet tip and transferred into a fresh tube containing 70 % ethanol. After pelleting again, the DNA was dried at room temperature and finally resuspended in TE buffer. RNase was added to a final concentration of 1 mg/ml to remove any contaminating RNA.

6.3.2 Polymerase chain reaction

Amplification of a particular DNA sequence from genomic DNA was performed by means of the polymerase chain reaction (PCR). Standard PCR reactions contained 1x PCR buffer according to the polymerase, 0.8 mM dNTP, 0.4 µM primers, 1-2 U polymerase, 5 % DMSO, approx.300 ng genomic DNA template and sterile Milli-Q water. PCR reactions were performed using the GoTaq DNA polymerase from Promega for analytical purpose and the proofreading Phusion High-fidelity DNA Polymerase from NEB for cloning purposes.

The amplification was routinely performed in the T3 Thermocycler (Biometra, Göttingen, Germany) and PCR conditions were chosen as follows: Initial denaturation 95°C for 5-6 min; denaturation step 95 °C for 30 s; annealing step at variable temperature for 30 s; elongation step at 72 °C for variable time; final elongation at 72 °C for 5 min.

6.3.3 Restriction analysis

For analytical restriction analysis, approx.200 ng of DNA was incubated with 1-5 U enzyme and 10 X buffer in a 10 µl volume. For preparative DNA restriction, 1-5 µg of DNA were incubated with 1-5 U enzyme and buffer in a total volume of 30 µl at 37 °C

for 1-2 hours. For cloning purposes, plasmids were dephosphorylated with TSAP (Thermosensitive alkaline phosphatase; Promega) after restriction and the enzyme was inactivated at 65 °C for 15 min, before the plasmid was purified by agarose gel extraction.

6.3.4 Ligation of insert and vector DNA

For ligation, insert and plasmid DNA after restriction digestion were mixed in a ratio of 3:1 and was incubated with 1-3 U of T4 DNA ligase (Roche) and buffer at 16 °C overnight.

6.3.5 Preparation of electrocompetent *E. coli* cells

For the preparation of electrocompetent *E. coli* cells, cultures were grown at 37 °C in LB to exponential growth phase, harvested and resuspended in ice cold 10 % glycerol. After washing twice with 0.2 volumes of ice cold 10 % glycerol, cells were resuspended in 300 µl of 10 % glycerol, aliquoted and stored at -80 °C.

6.3.6 Electroporation of *E. coli*

5-10 µl ligation reaction was mixed with 50 µl competent *E.coli* cells and transferred to a 2 mm ice-cold electroporation cuvette. A short electric pulse (settings: 25 µF; 200 Ohm; 2.5 kV on a Bio-Rad GenePulser) was applied to the mix and 1 ml warm SOC medium added quickly, transferred to a microfuge tube and incubated at 37 °c with shaking for 1 hour. Positive clones were selected using appropriate antibiotics.

6.3.7 Conjugation

For the cloning of plasmid DNA into *Burkholderia* strains, Triparental mating according to de Lorenzo (de Lorenzo & Timmis, 1994) was employed. The conjugation was performed with *E. coli* Top 10 strains harboring the plasmid of interest (donor), *E. coli* pRK600 or pRK2013 providing the *tra* genes for conjugation (helper) and the *Burkholderia* strain that will take up the plasmid (recipient). The cultures of respective strains were grown to mid-exponential phase, centrifuged at 6000 rpm for 5 minutes to pellet the cells and finally re-suspended in 1 ml LB. 200 µl of each culture was mixed in one tube while a reaction control was set up from which the donor strain had been excluded. 400 µl of the suspension from test and

control tubes was dropped onto LB plates and incubated at 37°c. After 5 hours, the cells were removed from the plate after pouring 1 ml of LB medium upon them and plated on selective PIA plates.

6.3.8 Sequencing

DNA Sequencing was performed according to Sanger's dideoxynucleotide chain termination method (Sanger *et al*., 1977) using the in-house sequencing service on the ABI 3730 DNA sequencer at the Institute of Plant Physiology (University of Zurich, Switzerland) or sending the samples to Microsynth AG (Balgach, Switzerland). For in-house sequencing, the samples were prepared as described below.

ABI Big Dye®	0.8 µl
5x Sequencing buffer	1.5 µl
Primer (5 pmol/µl)	0.25 µl
DNA (100 ng)	1.0 µl
ddH_2O	6.45 µl

Thermocycler program:

Initial denaturation: 94 °C for 2 min

Denaturation:	96 °C for10 s	60X
Annealing:	according to primer Tm for 5S	
Elongation:	60 °C for 3 min	

Sequences obtained after sequencing were analysed and confirmed using CLC main workbench

6.3.9 RNA extraction and DNAse treatment for qPCR

Strains to be tested were grown in appropriate conditions and then used resuspended in 100ml of respective medium at an OD_{600} 0.05. After growth to desired growth phase, 13.5 ml of the culture was rapidly transferred to cold tubes

containing 1.5 ml of “stop solution” (10 % phenol buffered with 10 mM Tris-HCl, pH 8, in ethanol), centrifuged 5 min at 5000 rpm at 4 °C and the pellet frozen in liquid nitrogen. Total RNA was extracted using a hot acid phenol protocol. Briefly, the pellet was resuspended in 1.5 ml ice cold buffer A (20 mM sodium acetate pH 5.5, 1 mM EDTA) and added to a mixture of 160 µl 10 % SDS, 2 ml buffer A and 3.5 ml acid phenol. The suspension was vigorously mixed for 30 s and incubated for 7 min at 65 °C with an additional vortexing step of 1 min in between. The aqueous phase was separated and re-extracted twice; first with 3 ml phenol/chloroform/isoamylalcohol and then with 2.5 ml chloroform. The total RNA was then precipitated at -80 °C overnight with 1/10 volume of 3 M sodium acetate pH 5.5 and 2 volumes of 100 % ethanol.

Quality control for the RNA was performed using RNA Nano Chip (Agilent 2100 Bioanalyzer; RIN >8). To remove the 5S rRNA that would interfere with the subsequent mRNA enrichment procedure, 50 µg of total RNA in a volume of 100 µl was run using columns of the RNAeasy MiniKit (Qiagen), according to the manufacturer’s protocol, and eluted twice, each time with 30 µl of elution buffer.

To further deplete the DNA, 30 µg of the RNA was treated with RQ1 RNAse-Free DNAse I (Promega) and SUPERase in RNAse Inhibitor (Ambion) for 30 min at 37 °C and purified with the RNAeasy MiniKit (Qiagen). The successful depletion of DNA was confirmed by PCR using primers ftrA_fwd and ftrA_rev (target: ftrA) with 35 cycles.

6.3.10 qPCR analysis

The expression of different genes were analyzed with an Mx3000P instrument using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent, Switzerland) and cDNA prepared from biological replicates as template. Each reaction contained 12.5 µl 2× Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix, 0.7 µM of individual primers and 15 or 7.5 or 3.5 ng of cDNA in a total volume of 25 µl. Reactions were run in triplicates. The relative expression ratio was calculated according to Pfaffl (Pfaffl, 2001)using the primary sigma factor *rpoD* (*BCAM0918*) or *recA* as a housekeeping gene.

6.3.11 Fur titration assay

Fur titration assay (FURTA) enables the identification of fur regulated promoters and iron binding proteins by introducing a multicopy plasmid carrying the promoter of interest into *E. coli* H1717. *E. coli* H1717 carries a Fur regulated *fhuF*:: *lacZ* fusion which is sensitive to changes of iron concentration in the medium. Upon introduction of a multicopy plasmid carrying a promoter with the Fur box nucleotide sequence into *E. coli* H1717, the multiple Fur boxes bind to Fur protein and deplete the Fur protein pool. Consequently, this leads to derepression of the *fhuF-lacZ* fusion, leading to transcription of the *lacZ* gene resulting in a lac+ phenotype in MacConkey plates. In contrast, colonies with plasmids that do not contain a Fur-regulated promoter appear white.

FURTA was performed as previously described. Approximately 1.3kb *ftr* promoter region upstream of *ftrA* was cloned into the high copy number plasmid, pBluescriptII KS and introduced by transformation into *E. coli* Top 10, which was then transferred into *E. coli* H1717. The clones were plated onto the differential medium, MacConkey agar containing 40 µM $Fe(NH_4)_2(SO_4)_2$. H1717 with the plasmid p3ZFBS containing the *E. coli* consensus Fur box and H1717 containing the empty plasmid pBluescript II KS were used as positive and negative controls respectively. Dr. Mark S Thomas, University of Sheffield, kindly provided *E. coli* H1717 and the positive control plasmid.

8.4 Phenotypic characterization of mutants

8.4.1 CAS assay

To characterize and analyze the siderophore production pattern of different strains, CAS assay was performed. 5 µl of overnight culture of bacterial cultures were dropped on CAS plates and incubated overnight. Siderophore production was determined by measuring the orange halo around the colonies. To detect siderophore activity in spent culture medium, equal parts of spent cell-free culture medium and CAS assay solution was mixed and incubated at room temperature for approximately 30 minutes. Generation of orange color was monitored and quantified at 630 nm by comparing with a blank containing sterile medium.

8.4.2 Analysis of pyochelin production

The analysis of pyochelin production in liquid cultures was based on the method of Sokol (Sokol, 1984) and Visca *et al*. (Visca *et al*., 1992). Strains were grown in iron free succinate medium at 37 °C with aeration for 40 h. Cells were collected by centrifugation, and the spent culture supernatants were filtered, acidified, and extracted with 0.4 volume of ethyl acetate. The organic phase was evaporated to dryness and the residue was resuspended in 40µl of methanol. The extracted pyochelin was analyzed by chromatography on a thin (0.2-mm) layer of silica gel 60 (Merck) with chloroform-acetic acid-ethanol (90:5:2.5 [vol/vol]). After developing the plate, ferric chloride was sprayed to visualize the siderophores.

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9. Appendix

9.1 List of figures and tables

Figures

Tables

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